GROWTH STUDIES WITH RATS KEPT UNDER CONDITIONS WHICH PREVENT COPROPHAGY[1]

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TWO FIGURES

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In present day nutritional studies involving rats it is possible to prepare highly purified rations of known composition and to select animals from well standardized strains. In this manner some of the more troublesome variables of earlier work are successfully avoided. However, there remain certain factors in all such experimentation which are not so easily controlled. Probably the most important of these is the influence of the intestinal flora, which is free to operate in 2 distinct ways. First, in a direct manner where the products of bacterial synthesis or degradation are absorbed through the intestinal wall of the rat; and second, in an indirect manner where the feces or urine are ingested by the rat. Since both feces and urine contain products of the animal's own metabolism as well as substances of bacterial origin, it is obvious that the animal's own secretions and excretions are a

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source of variation in nutritional studies. No matter how pure the ration may be or how clean the ordinary type of cage is kept, there always remains the possibility that the animals can obtain contaminating natural products in the form of feces, urine, skin secretions, and hair.

To control the direct effects of the intestinal bacteria, it is necessary to effect a change of some sort in the flora itself. This can be done by the use of bacteria-free animals, or by the easier practice of feeding substances which either alter the kind and strain of bacteria present or else react with compounds produced by the flora. Thus, by the feeding of sulfadrugs it can be shown that the rat requires a dietary source of biotin (Nielsen and Elvehjem, '42), folic acid (Daft and Sebrell, '43), and vitamin K (Black et al., '42). By the feeding of avidin the rat can be shown to require dietary biotin (Eakin et al., '40).

The control of the indirect influence of the bacterial flora and the animal's own secretions can best be exercised by preventing the animal from eating its feces and urine, and from licking its hair coat. Osborne and Mendel ('11) made the observation that rats allowed to eat their feces grew better than those not permitted to do so. Steenbock et al. ('23) reported that animals receiving feces and half the required amount of vitamin "B" grew much better than those given no feces. That rats would eat 44 to 100% of their feces offered in the dried state was reported by Roscoe ('31), who also showed that rats lived longer on vitamin "B"-free rations when allowed to eat their feces. The work of Dutcher and Francis ('23) on refection caused by feeding raw starch clearly indicated the importance of coprophagy. Though it would appear that the practice of using raised screen floor cages should eliminate coprophagy, one frequently sees animals kept under such conditions obtain feces pellets as they leave the body. It is also very common to see these animals lick themselves and their cages after urination. These observations have led various investigators to attempt the construction of devices designed to prevent coprophagy.

The restriction of the rat's movements by means of a harness has been unsuccessful to date because such mechanisms must be held tightly in place but not so rigidly as to annoy the animal. Furthermore, the harness method of confinement does not prevent the animal from going around the cage and licking off the adhering fecal, urinary, and epidermal residues. To surmount these difficulties investigators have tried to construct coprophagy-preventing cages.

Schwartzer ('37) constructed a cage which was in effect a stationary harness. It consisted of a large sized test tube rack with enlarged holes and which was turned on its side when in use. The animal was so placed that the head, neck, and forepaws passed through the hole, and adhesive tape was used to keep the rat in this position. In these cages weanling rats were reported to survive for an average of 10 days when given a diet of raw whole milk. These rats died before developing anemia, and on autopsy it was found that the gastrointestinal tract was bloody. Comparable animals kept under these conditions but fed their own feces and urine in addition to the milk survived an average of 20 days at which time they showed a hypochromic anemia, but no bloody intestinal tract. No minerals were added to the milk.

Mannering and Cannon ('44) constructed a cage which consisted of an endless circular runway formed by the space between a screen wire bottom and a sheet metal cover which were separated by 2 perpendicular metal bands of unequal diameter. These workers reported no studies in which this cage was used.

Geyer and Boutwell ('45) developed a tubular cage for metabolism studies to prevent any contamination of the excreta by the food. A tube of wire mesh just large enough to hold the rat was attached to a small wooden shaft. A food cup and water bottle were provided. Animals about 200 gm in weight were used and the collection periods were of short duration. Although a number of results were obtained, no growth studies were attempted.

The purpose of this paper is to describe several models of coprophagy-preventing cages and to report some of the nutritional experiments performed with them.

EXPERIMENTAL

Two types of coprophagy-preventing cages were constructed. One was a circular type which allowed the animal an endless circular runway. The other was a tubular type which held the animal in a somewhat fixed position. A detailed description of each and the nutritional studies made with each follow.

The circular type cage

The circular type of cage (fig. 1) consisted of a raised floor (A) made of a disc of metal screening with vertical sheet metal sides (B). One of 2 adjustable metal bands ($\frac{3}{4}$ or $1\frac{1}{2}$

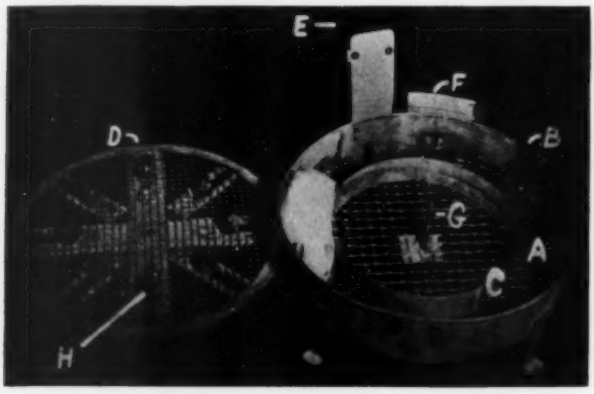

Fig. 1 Circular type anti-coprophagy cage.

inches in height) formed the inner boundary circle (C) and was held in place by a circular screen top (D) which could be raised or lowered to fit the rat. Because of the adjustable width and vertical height, rats varying from 40 gm to 200 gm could be accomodated easily. A water bottle (E) and removable food cup (F) were provided. The rat was placed in

the circular runway and the inner band was enlarged until the animal had a minimum amount of room. The cover was then placed on top and fastened securely by means of the screw adjustment (G). Bolts (H) protruded through the spaces provided in the cover, and by means of the wing-nuts provided the band was held in place. Further adjustments were made as warranted by the growth of the confined animal.

TABLE 1

Experimental rations.

	BASAL RATION	
	I	*II*
Sucrose	76	..
Lactose or sucrose	..	48
Casein [1]	18	20
Salts IV [2]	4	4
Corn oil [3]	2	..
Corn oil or butter fat	..	28

Vitamins added per 100 gm ration:

Diet I: 300 μg thiamine; 400 μg riboflavin; 350 μg pyridoxine; 300 μg nicotinic acid; 200 μg 2-Me-1, 4-naphthoquinone; 7 μg biotin; 2.5 mg Ca pantothenate; and 100 mg choline chloride.

Diet II: Same as for Diet I except for the following: 150 mg choline chloride; 210 μg 2-Me-1, 4-naphthoquinone; 14 μg calciferol [4]; 560 μg beta-carotene [5]; and 2.24 mg alpha-tocopherol.

[1] Extracted for three 2-hour periods with boiling alcohol.

[2] Hegsted et al. ('41).

[3] Mazola brand. Corn Products Refining Co.

[4] Crystalline irradiated ergosterol.

[5] 90% β-carotene and 10% α-carotene.

Twenty-one-day old male rats of the Sprague-Dawley strain weighing approximately 42 gm were used in all of the nutritional studies. Two groups of 3 rats each were placed on experiment in these cages and 2 identical control groups were kept in the usual screen floored dormitory type cages. All feeding was ad libitum. Three rats in each type of cage received the Basal Ration I given in table 1. Each animal received 3 mg of alpha-tocopherol per week in 2 drops of a

1:6 dilution of haliver oil in corn oil. Three rats in each type of cage received the same basal ration plus 2% solubilized liver [2] added at the expense of the entire ration (see table 2). The animals were weighed at weekly intervals and hemoglobin values and both differential and total white cell counts were made at intervals. The data obtained are shown in table 2.

TABLE 2

Results of the experiments with the circular cages. (Each figure represents the average of 3 male rats).

CAGE	RATION	GAIN IN 6 WKS.	HEMOGLOBIN	TOTAL WHITE CELLS	DIFFERENTIAL COUNT
		gm	*(gm/100 ml)*		
Circular	Basal no. I	106	13.38–14.08	7,000– 9,400	Lymph. 75 Neutr. 25
	Basal no. I + S.L.P.[1]	189	13.77–14.08	13,800–17,200	Lymph. 78 Neutr. 20 Monocyte 2
Square — "dormitory type"	Basal no. I	167	12.91–14.28	9,000–15,600	Lymph. 76 Neutr. 24
	Basal no. I + S.L.P.	210	12.91–14.08	13,800–16,000	Lymph. 80 Neutr. 20

[1] Wilson solubilized liver powder (Fraction "L").

The tubular type cage

Two different types of tubular cages were constructed. The first consisted of a cylinder ½-inch mesh wire screen attached to screen wire supports which enabled the hanging of the cage from overhead rods. The cylinder itself was a tube whose longitudinal edges overlapped to allow for expansion as needed. A food cup was held at the front end of the cage by means of a loop of screen wire, and the animal was kept from backing out of the cage by means of a U-shaped wire pin. A water bottle was slung from a wire loop. After several experiments were conducted in this type of cage, several

[2] Wilson's Liver Fraction "L."

mechanical difficulties were found which made construction of another type desirable.

The second type of tubular cage had as its basis the spiral of wire screening with the edges which overlapped covered with strips of metal to prevent injury to the rat. The spiral (A) was attached to a sheet metal support (B) which was cut from one piece of metal in the design shown in figure 2. The food cup (C) slipped into the metal holder (D) in front of the spiral and was held there by tension. When used with weanling rats, a Gooch crucible was placed in the food cup opening

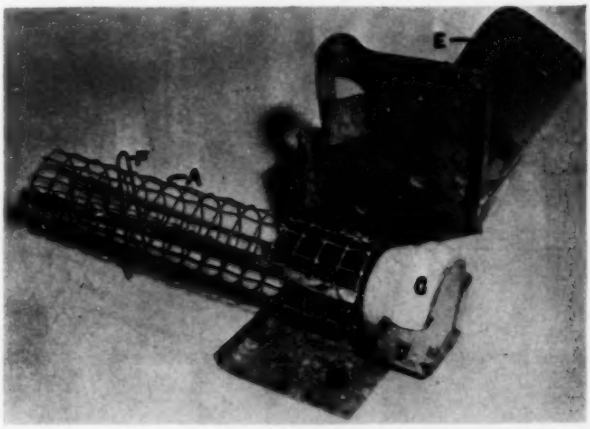

Fig. 2 Tubular type anti-coprophagy cage.

to prevent the small animal from living in the food cup. Such a crucible was found to hold enough food for daily feedings up to the end of the first week of the experiment. The water bottle (E) was held in such a position as to prevent the rat's fur from rubbing on its open end and causing leakage which was found in earlier experiments to affect growth. A U-shaped pin (F) served to keep the animal from backing up. Part of the support was bent to provide a shelf (G) under the front end of the spiral and served to catch the spilled food. The entire cage was suspended from a metal angle iron which

TABLE 3

Results of experiments with the tubular cages.

EXPT. NO.	CARBOHYDRATE	CAGE	FAT		LIVER [1] ADDED	FECES GIVEN	GM [2] GAIN IN 6 WEEKS
			BF [3]	CO [4]			
1	Lactose	Tube	+		−	−	80
				+	−	−	55
			+		+	−	118
				+	+	−	84
		Square dormitory type	+		−	−	142
				+	−	−	140
			+		+	−	146
				+	+	−	136
2	Sucrose	Tube	+		−	−	95
				+	−	−	77
			+		+	−	157
				+	+	−	129
		Square dormitory type	+		−	−	161
				+	−	−	165
			+		+	−	220
				+	+	−	176
3	Sucrose	Tube	+		−	−	121
			+		−	+	104
				+	−	−	77
				+	−	+	81
			+		+	−	155
			+		+	+	153
				+	+	−	124
				+	+	+	123
		Square dormitory type	+		−	−	177
			+		−	+	145
				+	−	−	122
				+	−	+	128
			+		+	−	190
			+		+	+	183
				+	+	−	156
				+	+	+	151

[1] Wilson Fraction "L."

[2] Each figure represents the average of 3 male rats.

[3] Butter fat.

[4] Corn oil.

was large enough to accommodate 4 such cages. To obtain samples of excreta, a low wide dish covered with a metal screen was placed under the rear of the cage.

Weanling male rats of the Sprague-Dawley strain weighing approximately 42 gm were used in these experiments. They were fed the Basal Ration II given in table 1. The carbohydrate portion was either lactose or sucrose, and either butter fat [3] or corn oil was used as the fat. The basal ration was fed with or without the addition of 2% of solubilized liver powder added at the expense of the entire ration. The groups of animals used and the growth responses in 6 weeks are given in table 3. In the second sucrose experiment 3 rats in each group of 6 were fed feces of the animals on the same ration which were housed in the dormitory type cage. Initially 500 mg of wet feces were fed to each animal per day, but the amount was lowered gradually until they were receiving 100 mg per day each. The results of this forced coprophagy are also given in table 3.

RESULTS

When rats were placed in the round cages and fed Basal Ration I, without liver, their growth was sharply curtailed as compared with control rats receiving the same ration but kept in dormitory type cages. As is shown in table 2, when the liver concentrate was incorporated into the ration, the confined animals grew almost as well as the supplemented rats in the square cages. The experiment was repeated with similar results. The rats in the round cages showed normal hemoglobin values but slightly reduced white cell counts.

Rats were kept successfully in the tubular type cages for 6 or more weeks and as shown in table 3, their growth on Basal Ration II containing no liver supplement was poor on either lactose or sucrose. Liver powder was only partially effective in maintaining good growth under these conditions. The animals in the tube cages which received butter fat grew

[3] Prepared from fresh unsalted University Creamery butter by heating at 60–70°C. and decanting from the water and curd.

better on all regimens than the rats fed the corn oil rations. In Experiment 3 the animals fed feces did no better than those fed none. There were a number of animals which showed a watery eye condition and an occasional one that would have tightly closed eye-lids because of the drying of the exudate. Some of the rats developed an eye condition which resembled the "spectacled eye" condition seen in biotin deficiency. All of the animals confined to the tubular cages developed a marked tendency to lick anything within reach of the tongue. Feces feeding did not stop this abnormal behavior.

DISCUSSION

The results obtained with the circular and tubular cages demonstrated that weanling rats will grow under conditions of severe confinement. Their growth was, however, below that of rats fed the same rations but kept in dormitory-type cages. Rats in the latter type of cage grew well with or without liver concentrate in the ration, but the inclusion of the liver gave a definite increase in growth except in the case of the animals fed the lactose diet. In the case of the animals kept in the circular cages very poor growth was obtained without the liver concentrate, whereas, those receiving the liver gained 189 gm in 6 weeks or 83 gm more than those receiving no liver. Since the liver-fed rats in the square cages gained 210 gm during the same period, it seems evident that the importance of feces consumption in the presence of liver is not great. In the absence of liver the eating of feces and other excretory products is of importance since the ration containing no liver allowed 61 gm more gain in 6 weeks for the animals kept in the square cages than the comparable ones confined to the circular cages. Because all of the rations contained biotin, nicotinic acid, and vitamin K, these substances are not responsible for the effect of the feces unless still higher amounts of them are required. Whatever the missing substances are, it is evident that they do not play a role in maintaining either normal hemoglobin or white cell values,

at least under these experimental conditions over a 6-week period.

When animals are kept in the circular cage perfect anti-coprophagy conditions are not attained since the rat travels over screening which it has previously used. Thus, adhering fecal residues are easily licked off and even though the amount is insufficient, it introduces a variable which cannot be controlled. There is also the possibility that urinary and dermal residues are ingested and interfere with obtaining a definite deficiency. In cases where the animals develop diarrhea the circular cage is of little value.

The above difficulties encountered with the circular cage are completely overcome by means of the tubular cage. Whether this fact is reflected in the poorer growth of the rats confined to the tube cages must await further study. Since such animals were unable to get any body excretions except what might be on the forepaws, head, and neck, it would be expected that they would not grow as well in comparison to their controls as did the circular cage animals. Furthermore, the results show that the addition of liver powder to the ration aided the animals, but the growth in no instance was as close to that of the controls as in the circular cage experiment. It must be remembered that in these 2 studies the rations employed were not comparable, especially with respect to the level of fat. At present the effect of various rations under similar experimental conditions is being studied.

The tube cage study demonstrated that the carbohydrate portion of the ration was of importance because where lactose was used the growth was much poorer and the animals in many instances developed severe diarrhea. The growth of the animals in either the tubular or dormitory type cage was below that normally obtained on such diets. On the sucrose rations no diarrhea developed. The carbohydrate present in the studies of Schwartzer ('37) with raw whole milk was, of course, lactose, and this may have added to the poor performance of his rats with or without the feeding of feces. It should be noted that the lactose content of the ration em-

ployed in the work reported in this paper was higher than would be present in whole milk solids, yet the animals grew instead of died. Minerals were not added to the milk employed by Schwartzer, and their absence was probably responsible for the anemia which developed in the animals which survived the longest. It is also possible that the raw milk acted in a manner similar to that of raw starch and that the animals fed feces had the benefit of not only the iron and copper in the feces, but also of some factor or factors needed for survival on such a ration. In the few cases in this laboratory where the effect of the feeding of milk to animals confined in the tubular cages has been tried, the majority of them developed diarrhea even though they were older rats which had been on a milk diet previously to the study.

The data in tabe 3 show that in all 3 experiments the animals confined in the tubular cages grew better on the rations containing butter fat than on the corn oil diets. In the case of the animals kept in the ordinary dormitory cages this difference between fats was inconsistent. It is possible that the anticoprophagy conditions prevent the rats housed in the tubular cages from obtaining certain substances present in feces or urine which have an influence on the nutritive value of the dietary fat. Boutwell et al. ('45) have shown that the B-vitamins in the diet have such an influence.

The tendency of all rats in the tube cages to lick their cages and anything within reach of their tongue is suggestive of a deficiency. To date, the only deficiency in which this curious phenomenon has been reported in the rat is that of potassium (Orent-Keiles and McCollum, '41). It seems unlikely that this element per se is responsible for the condition in the experiments reported here, for it is supplied in liberal amounts in the salt mixture. The condition of the eyes of many of the animals must at present be regarded only as a symptom of unknown origin and must await further study. Biotin was provided, but it is possible that a higher level was required.

The early observations of Osborne and Mendel ('11), Steenbock et al. ('23), and other workers of the period (Kennedy

and Palmer, '28; McCollum et al., '25) cannot be readily compared with the studies reported in this paper for although these investigators reported the beneficial effects of coprophagy, they were working at a time when little was known of the individual vitamin requirements. Since they probably were dealing with inadequate rations, a large part of the influence of the feces could have been due to such vitamins as thiamine and riboflavin. It seems desirable to study with a complete synthetic ration the effect of keeping rats on sheet metal floored cages in contrast to the raised screening type to determine whether or not the added excreta which animals kept in the former type of cage can obtain, is of any importance.

The report of Schwartzer ('37) was based on an incomplete ration, and he was unsuccessful in obtaining healthy rats. Although his method of coprophagy prevention is simple, it seems less convenient than the tubular cage and especially so where excreta collections are desired.

Further studies are needed to determine what factors are required by the rat confined in either the circular or tubular cages. It is also important to ascertain, if possible, whether the probable decrease in exercise is of any real importance. Once these questions have been answered, the tubular cage would make an excellent metabolism cage, for contamination of the excreta by food or reingestion of materials through coprophagy is prevented.

SUMMARY

Two types of coprophagy preventing cages have been constructed and found to allow fair growth of rats over a 6-week period.

In the circular cage described, animals fed no liver concentrate grew poorly; whereas, those receiving such a supplement grew almost as well as control rats kept in ordinary dormitory cages. The imperfections in such a cage are discussed.

Rats confined in the tubular cages grew poorly without a liver supplement, but even when the latter was furnished, growth did not approach as closely to that of control animals

as was found in circular cage experiments. The animals showed a marked tendency to lick everything within reach; some of them had watery eyes and occasionally an eye condition resembling the spectacled eye of biotin deficiency. In all cases, the animals confined to the tubular cages grew better on rations containing butter fat than on comparable rations containing corn oil.

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COPPER AND MANGANESE STORAGE IN THE RAT, RABBIT, AND GUINEA PIG

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This study was undertaken to determine the storage of copper and manganese in the liver of 3 species, the rat, rabbit, and guinea pig, at various age levels, namely, newborn, 2 weeks, 3 weeks, 2 months, and adult. Analyses were made on the newborn in order to reveal the extent of placental transmission of copper and manganese, at 2 weeks as an indication of the influence of mother's milk since 2 of the 3 species investigated receive milk as their sole source of nutrients during this period, at 3 weeks, which represents the average weaning age, at 2 months, which approximates the period of sexual maturity, and at adult or full maturity.

Although studies of the storage of copper in the liver at various ages in any species are limited in number and involve few experimental subjects, there seems to be agreement that in most species there is a considerable supply deposited during intrauterine life.

Lindow, Peterson, and Steenbock ('29), in studying the metabolism of rats, observed that, as the animal increased in age, there was a continual storage of copper of about 0.001 mg per day for the first 12 days following birth and about 0.002 mg per day thereafter. They were unable to increase the copper content of the newborn rat by feeding the mother a copper-rich diet, indicating that there was neither increased placental

transmission nor increased transmission of copper through the milk.

Further review of the literature on the copper content of livers of small laboratory animals is given in the discussion.

Manganese is apparently transferred through the placenta if present in the mother's blood (Orent and McCollum, '31). A manganese-rich diet fed during gestation produced a noticeable increase in the manganese content of newborn rats, and postpartum supplementation considerably increased the body content of the element at 21, 70, and 180 days (Skinner, Peterson and Steenbock, '31). Manganese is stored slowly in young receiving only mother's milk.

EXPERIMENTAL

Animals

Regular stock colony animals (Dutch rabbits; Sprague-Dawley rats; U. S. Dept. Agric. family 13 inbred guinea pigs) were used in these experiments. The rats were fed dog pellets ad libitum. The rabbits and guinea pigs were fed a good quality mixed hay and a grain mixture consisting of 50 parts by weight of oats and wheat, ad libitum; they also had access to a salt block containing additional iron and iodine. On analysis, the mixed grain, dog pellets, and hay were found to contain 3.1, 12.9, and 5.1 μg copper per gm dry weight and 41.8, 35.0, and 70.7 μg manganese per gm dry weight, respectively.

Ten animals of each species, with the exception of the newborn rats, were etherized at each of the 5 age levels, and the livers removed and weighed immediately. Where possible, individual livers were used for analysis but in the case of the younger animals it was necessary to pool 2 or more in order to obtain a satisfactory size sample. In the case of the newborn rat, the liver was so small that approximately 30 livers were pooled for each sample; thus there are only 3 samples in this age group, representing approximately 100 animals.

The liver was chosen for analysis because of the general agreement (Bunge, 1892; Lindow, Peterson and Steenbock, '29; Cunningham, '31; and Chou and Adolph, '35) that it is a principal site of storage of both manganese and copper in the animal body.

Methods of analysis

Four-gram samples of fresh liver were placed in covered silica dishes, moistened with 2 ml of a 20% solution of $Mg(NO_3)_2$, and ashed in a muffle furnace at 450°C. for a period of 16–18 hours. No dish was used for more than 5 ashings because of the possibility of adsorption of copper (unpublished data) by etched silica. The samples were treated with 2 ml of a nitric-perchloric acid mixture (20:1 by volume), evaporated to dryness on a hot plate, and re-ignited until a white ash was obtained, which in some cases required a second treatment with nitric-perchloric acid. Each ash sample was extracted with three 5 ml portions of N nitric acid and made up to 25 ml volume. Manganese and copper were determined colorimetrically on aliquots of this extract. All analyses were run in duplicate, and unless they agreed within 2 transmission units, were repeated. Copper was determined by the sodium diethyldithiocarbamate method (Callan and Henderson, '29; McFarland, '32; Eden and Green, '40; and Sandell, '44) and manganese by the periodate method (Skinner and Peterson, '30), with slight modifications. The transmission values were read in a photoelectric colorimeter (5 cm absorption cells) using Corning filters 3389 and 5113 for copper, and 4303 and 3486 for manganese determinations. Excellent agreement was obtained in both methods using 2- and 4-gm samples run simultaneously in quadruplicate. Standard solutions of both elements were added separately to 3-gm samples in quadruplicate before ashing, and recovery values ranged from 99 to 102%.

The data obtained were reduced by analysis of variance and the t test used to determine whether particular differences were significant. Odds greater than 19:1 are accepted as being

significant, and odds greater than 99:1 are considered highly significant.

RESULTS

The copper and manganese contents of the livers are presented in 2 different ways — total amount in the liver and concentration. A very different picture of the storage of these elements is obtained depending upon the method of comparison that is used.

Copper

The mean totals and the mean concentrations of copper in the liver of the 3 species at the various age levels are presented in table 1. An analysis of variance of all the data is summarized in table 3. It will be noted that there are differences in both total amount and concentration of copper in the livers among species and among the age groups. These differences are highly significant. Specific differences which appear most important will be pointed out in the following discussion.

It will be noted in table 1 that the total copper of the livers varied greatly among the newborn guinea pigs, rabbits, and rats. These differences were a function of both size of the livers and the concentration of the elements. In the guinea pigs and the rabbits the total liver copper decreased significantly during the suckling period. Following weaning (3 weeks) the total copper increased progressively to reach the highest level in the adult animals. On the other hand, the total copper in the rat livers increased progressively from birth to the adult stages and showed no decrease during the suckling period. In all 3 species there was a very rapid increase in total copper between the ages of 3 weeks and 2 months. This probably is a reflection of the increased copper intake due to the replacement of the copper-poor milk diet with natural feeds containing more copper.

The concentration of copper in the liver presents a picture considerably different from that given by the total copper in

TABLE 1

Total amount and concentration of copper in the liver of guinea pigs, rabbits and rats at the various age levels (mean values with their standard errors).

AGE GROUP	GUINEA PIGS		RABBITS		RATS	
	Content	Concentration	Content	Concentration	Content	Concentration
	μg/liver	*μg/gm* [1]	*μg/liver*	*μg/gm*	*μg/liver*	*μg/gm*
Newborn	161 ± 11.7	67 ± 5.6	47 ± 5.8	37 ± 6.7	4 ± 0.3	58 ± 4.0
2 weeks	80 ± 10.7	26 ± 3.5	58 ± 10.2	21 ± 2.4	28 ± 3.2	62 ± 5.0
3 weeks	110 ± 7.0	21 ± 2.4	35 ± 2.2	12 ± 0.6	27 ± 1.9	20 ± 1.2
2 months	264 ± 31.3	19 ± 1.2	213 ± 44.8	24 ± 3.2	63 ± 2.5	9 ± 0.4
Adult	619 ± 92.1	23 ± 3.5	842 ± 98.1	23 ± 3.6	227 ± 17.9	34 ± 2.9

[1] Dry liver.

TABLE 2

Total amount and concentration of manganese in the liver of guinea pigs, rabbits and rats at the various age levels (mean values with their standard errors).

AGE GROUP	GUINEA PIGS		RABBITS		RATS	
	Content	Concentration	Content	Concentration	Content	Concentration
	μg/liver	*μg/gm* [1]	*μg/liver*	*μg/gm*	*μg/liver*	*μg/gm*
Newborn	3.1 ± 0.29	1.3 ± 0.13	3.0 ± 0.22	2.3 ± 0.17	0.1 ± 0.01	1.9 ± 0.18
2 weeks	10.8 ± 0.96	3.5 ± 0.23	9.4 ± 1.04	3.4 ± 0.23	0.7 ± 0.06	1.5 ± 0.17
3 weeks	23.4 ± 2.40	4.1 ± 0.30	8.2 ± 0.45	2.7 ± 0.13	3.4 ± 0.14	2.5 ± 0.14
2 months	60.2 ± 4.67	4.4 ± 0.18	32.6 ± 2.71	4.0 ± 0.32	19.2 ± 1.41	2.6 ± 0.14
Adult	108.7 ± 9.49	4.1 ± 0.45	74.8 ± 10.32	1.8 ± 0.13	18.8 ± 2.53	2.6 ± 0.20

[1] Dry liver.

the liver. The concentration of copper in the livers of the newborn rabbits was significantly less (99:1) than in either the guinea pigs or the rats. The guinea pigs and the rats did not differ significantly. In all 3 species the copper concentration decreased significantly (99:1) following birth to reach a minimum at 3 weeks of age in the rabbits and at 2 months in the rats and guinea pigs. The copper concentration found in the adult animals was considerably less than that observed in the newborn young although, as previously mentioned, the total liver copper was much greater in the adult than in the newborn in all 3 species. The copper concentrations in the adult guinea pigs and rabbits did not differ significantly, but the concentration in the adult rat was significantly higher than either. This difference may be due to the fact that the rat diet contained a higher concentration of the element than did the guinea pig or rabbit diet.

Manganese

The mean totals and mean concentrations of manganese in the livers of the 3 species at various age levels are presented in table 2. As in the case of copper, an analysis of variance of the data showed differences which were highly significant among both species and age groups in total amounts and concentrations of the element in the livers.

As a general observation it will be noted that total amounts and concentrations of manganese in the livers are appreciably less than the respective copper values. All 3 species were born with a relatively low content and concentration of manganese. In all 3 species the total amounts of manganese increased in the livers progressively from birth onwards to reach a maximum at the adult stages in guinea pigs and rabbits, and at 2 months in the rats. The total amount of manganese as well as the total amount of copper increased rapidly between the ages of 3 weeks and 2 months in all 3 species.

The manganese concentration in the livers of the 3 species differed significantly at birth, the rabbits having the highest

TABLE 3

Analyses of variance of concentration and total amount of copper and manganese at various age levels.

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE			
		Copper		Manganese	
		Content	Concentration	Content	Concentration
Total	141				
Species	2	385,076 [1]	1,145 [1]	11,176 [1]	16 [1]
Age groups	4	1,315,884 [1]	5,722 [1]	21,057 [1]	12 [1]
Species X age	8	213,976 [1]	1,740 [1]	3,798 [1]	7 [1]
Error	127	15,497	120	172	0.5

[1] Significant difference at odds greater than 99 : 1.

TABLE 4

Live animal weight, fresh liver weight, and percentage dry matter of the liver of the 3 species (mean values with their standard errors).

AGE GROUP	LIVE ANIMAL WEIGHT (gm)			FRESH LIVER WEIGHT (gm)			PERCENTAGE DRY MATTER		
	G. pigs	Rabbits	Rats	G. pigs	Rabbits	Rats	G. pigs	Rabbits	Rats
Newborn	81 ± 3.0	44 ± 0.6	6 ± 0.1	5 ± 0.1	2 ± 0.1	0.2 ± 0.01	64 ± 1.2	67 ± 0.9	63 ± 0.9
2 weeks	116 ± 5.4	152 ± 12.3	22 ± 1.2	5 ± 0.1	5 ± 0.5	0.7 ± 0.06	76 ± 0.4	70 ± 0.8	74 ± 0.7
3 weeks	190 ± 17.1	166 ± 20.6	47 ± 1.3	9 ± 0.8	6 ± 1.0	2.0 ± 0.10	75 ± 0.4	76 ± 0.8	74 ± 0.2
2 months	420 ± 24.8	327 ± 25.6	188 ± 9.0	20 ± 1.1	12 ± 1.0	11.0 ± 0.60	74 ± 0.3	73 ± 0.4	74 ± 0.2
Adult	918 ± 40.2	2,367 ± 83.3	261 ± 18.7	38 ± 1.3	61 ± 4.8	10.0 ± 1.10	76 ± 0.5	69 ± 1.6	73 ± 0.4

concentrations. The range of variability of manganese concentrations within and between the species were of a relatively low magnitude as compared to copper as well as to many other minerals reported in the literature. The concentration in the livers of guinea pigs increased progressively following birth to reach a maximum at 3 weeks of age. In the rats the concentration decreased during the period of suckling, but not significantly. It reached the maximum value at 3 weeks of age. The mean concentrations of manganese in the rabbit livers in the various age groups were very variable for unexplained reasons. The mean manganese concentration in the adult rabbit livers was significantly less than in either the guinea pigs or the rats.

Table 4 summarizes the live weights, fresh liver weights, and the percentage of dry matter in the livers of the animals used in this study.

DISCUSSION

The data presented indicate that all 3 species, guinea pigs, rabbits, and rats, are born with a reserve store of copper in the liver which is drawn upon during the suckling period. Although the concentration of copper in the livers of the rats decreased during suckling the total amount increased indicating that the rat receives a substantial amount of copper from the milk of the dam. This observation that the milk of rats is relatively rich in copper is supported by the findings of Cox and Mueller ('37) who found rat milk to contain about 10 times as much copper as cow's or human milk.

In contrast to the observations on copper, the data show that the 3 species studied are born with relatively small amounts of manganese in the livers. In all cases the total amount and concentration of manganese in the livers increased progressively from birth onwards, particularly during the period in which substantial amounts of solid feed were consumed. From these observations it cannot be decided whether the small amounts of manganese in the livers at birth of the 3 species indicate a low reserve of manganese or not. Until

more is known of the manganese requirements of young animals the question must remain unanswered. The concentration of manganese in the livers of the species studied varied relatively little during the life span. This observation apparently also holds true for man, for according to Sheldon ('32), "There is no reserve store of manganese provided during fetal life to tide the infant over the nursing period. The manganese content of the liver rises during fetal life and reaches its maximum during the last 3 months, but the maximum is the normal adult level of about 0.0008% of the dry tissue; and once this has been obtained it apparently remains at this figure throughout life."

Many of the studies of nutrient reserves reported in the literature have been concerned only with the concentration of the nutrient in tissues. It will be noted in this study, as well as others previously reported, that a very different picture may be obtained depending on whether one uses total amount or concentration of the nutrient as the criterion of reserve stores. In some cases a decrease in reserves may be indicated by a study of concentration whereas the total amount of the nutrient may actually be increased. Whether concentration or total amount of a nutrient is used is particularly important in studying various age groups in which a difference in size of the liver is a variable influencing particularly the concentration. It is important that care be used in choosing the proper criterion for the particular study undertaken. It would appear that in most cases both total amount and concentration of the nutrient should be determined. The now classic paper of Lintzel and Radeff ('31) is a good example of the use of concentration and total amount of a nutrient (iron) in evaluating the reserve stores of animals.

A comparison of the values which we have obtained for the copper content of the livers of the various animals is compared with values reported by others in table 5. In general there is good agreement in the trends in concentration although the magnitude of concentration varies somewhat. The latter variation may well have been due to the feeding of diets

differing in copper content. For the most part the copper content of the diets has not been reported. A comparison of values of total liver copper as well as the manganese contents of livers of animals is not feasible due to the lack of information.

TABLE 5

Summary of studies on copper in livers recorded in the literature. All data are in mg/kg of dry matter.

AUTHOR	NEWBORN	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	8 WEEKS	ADULT
Guinea pigs							
Adrianoff and Ansbacher ('30)	50						16–20
Cunningham ('31)	149 203	59.6	30.8				17
Flinn and Inouye ('29)	28.4						37
Lorenzen and Smith (this report)	67.2		26.0	20.9		18.8	23.4
Rabbits							
Cunningham ('31)	5.5						9–10
Lorenzen and Smith (this report)	37.4		21.0	11.6		24.0	22.8
Rats							
Cunningham ('31)	87.8	23.5	14.5	17.8			10–12
McFarlane et al. ('32)	96–121				29–42		
McHargue ('25)	30.3						18.8
Lindow et al. ('29)	70						11.4
Lorenzen and Smith (this report)	58.5		61.6	19.8		8.6	34.2

SUMMARY

Guinea pigs, rabbits, and rats are born with a reserve store of copper which is drawn upon during the suckling period. In rabbits and guinea pigs both total amount and concentration of copper in the livers decreased during the suckling period. On the other hand, rats showed an increase in total copper in the livers from birth onwards even though the concentration decreased during suckling.

In all 3 species studied, the amount of manganese in the livers at birth is relatively low. Both total amount and concentration of manganese in the livers increased from birth throughout life, although the variation in magnitude of concentration of manganese in the livers was relatively small.

It is emphasized that in studies of nutrient storage in animal tissues the determination of only the concentration of nutrients may be insufficient.

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SOME PHYSIOLOGICAL RELATIONSHIPS OF PROTEIN, FAT, CHOLINE, METHIONINE, CYSTINE, NICOTINIC ACID AND TRYPTOPHANE [1]

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In 1915, Osborne and Mendel reported that diets containing 15 to 18% of casein along with essential nonprotein components produced normal growth in young rats; when similar diets containing 9 to 12% of casein were fed, growth was definitely limited but could be significantly increased by the addition of cystine. Jackson and Block ('32) later found that methionine also supplemented low levels of casein. Still later Womack and associates ('37, '41) concluded that methionine could replace cystine entirely and that the latter was effective only in the presence of a certain minimum amount of methionine. These findings have extended rather than questioned the original observations of Osborne and Mendel, which have been confirmed by other investigators.

In work relating to the physiology of choline and the B vitamins in 1941, however, it was observed in this laboratory [2] that growth was decidedly subnormal on low-fat, low-casein diets, and was depressed rather than increased by the addition of cystine. A reinvestigation of the nutritive value of casein was therefore undertaken. The results show some interesting relationships of protein, fat, certain amino acids, and vitamins.

[1] Published with the permission of the director of the Alabama Agricultural Experiment Station.

[2] Unpublished data by the author.

METHODS AND RESULTS

General procedure

A line-bred strain of hooded rats from the Wisconsin original stock was used throughout this investigation. Rats were weaned at 23 days of age and caged individually in metal cages with raised screen bottoms. Fresh tap water and food were supplied daily, ad libitum. Once a week all food residues were weighed and discarded and clean food jars were placed in the cages. The floor screens and the papers in drip pans were changed each week. The rats were weighed weekly at approximately the same time of day. Each group, with 5 exceptions, consisted of 2 males and 2 females, and the groups being compared always represented uniform litter composition. For economy of space the comparable control groups have been combined in tables 3 and 4.

The composition of the various basal diets is given in table 1. Adequate amounts of α-tocopherol, carotene, calciferol, thiamine, riboflavin, pyridoxine, inositol, and Ca pantothenate were mixed into the basal diets. Choline chloride was omitted from the diets used in the experiments listed in table 2 and for certain groups that received methionine as indicated in tables 3 and 4.

Special supplementary additions and major changes of diet are shown in tables 2 to 5.

Primary deficiency of casein is labile methyl

Much work has been done on choline-deficient diets containing various levels of casein. The results have shown that casein has lipotropic and kidney-protecting properties. Griffith's ('41) review gives a bibliography of the important work to 1941; see also Treadwell et al., '42, Beveridge et al., '44, and Handler, '46. Some of the results have been complicated by the inclusion of other proteins in casein diets and by the use of yeast or yeast extracts as sources of vitamins.

In the beginning of this study, rats that received choline-free diets containing crystalline vitamins and low levels of casein

TABLE 1

Composition of basal diets. Major ingredients and percentages thereof.[1]

DIET NUMBER	CASEIN [2]	SALT [3]	CARBOHYDRATE [4]	FAT [5]	CHOLINE [6]
6	6	3	90.8		0.2
8.3 L	8.3	3	58.5	30	0.2
9	9	4	86.8		0.2
12	12	4	83.8		0.2
12 L/2	12	4	68.8	15	0.2
12.5 L	12.5	4	53.3	30	0.2
15	15	4	80.8		0.2
16.5 L	16.5	4	49.3	30	0.2
18	18	4	77.8		0.2
18 L/2	18	4	62.8	15	0.2
18 L	18	4	47.8	30	0.2
24	24	4	71.8		0.2
24 L/2	24	4	56.8	15	0.2
25	25	4	70.8		0.2
25 L	25	5.5	39.3	30	0.2
30	30	4	65.8		0.2
30 L/2	30	4	50.8	15	0.2
41 L	41	5.5	23.3	30	0.2

[1] Additions of special supplements as noted in later tables were always at the expense of the carbohydrate component and were always in addition to the regular vitamin supplements carried by all diets in the following amounts in mg/kg of diet: carotene 4, a-tocopherol 50, calciferol 0.125, i-inositol 200, thiamine 2, pyridoxine 2, riboflavin 4, Ca pantothenate 10. Nicotinic acid was added only in certain groups as indicated in tables 2 and 5. The calciferol was donated by Mead Johnson Co., Evansville, Ind.

[2] The casein was purified in 50-lb. lots by cold percolation with tap water for 6 days. The water standing in the casein overnight was acidified to 0.20% with acetic acid. On the seventh day the acid was washed out and about 4 gal. of alcohol percolated through the casein to facilitate drying under a fan in a warm room.

[3] In the early experiments Salt 186 (J. Biol. Chem., 1930, vol. 89, p. 199) was used. In the later experiments Salt 5 was used. The composition of Salt 5, as mixed, was $Ca_3(PO_4)_2$ 2050, K_2HPO_4 1030, $MgSO_4 \cdot 7H_2O$ 600, NaCl 500, Fe citrate 130, $MnSO_4 \cdot 4H_2O$ 20, $CuSO_4 \cdot 5H_2O$ 5, $ZnCO_3$ 10, KI 5 gm. This was dried at 70°C. to a dry weight of 4000 gm.

[4] Sucrose (standard granulated cane sugar) was the carbohydrate used except for a few groups where it was replaced with glucose (cerelose) or cornstarch as shown in tables 3 and 4.

[5] Lard was the fat used except for 7 groups as shown in table 4. In all cases where the diet did not contain added fat, each rat received 0.10 ml/day of corn oil as a source of essential fat acids.

[6] Choline was omitted and the carbohydrate increased 0.20% for all groups listed in table 2.

died. The studies on the choline-free diets were extended. From the results shown in table 2, it is evident that the primary deficiency of casein at low levels is labile methyl. Kidney lesions and death occurred even at casein levels of 18 and 24% in choline-free diets containing a minimum of fat and no added cystine. At levels of 9, 12, and 15%, kidney lesions and mortality ranged from 75 to 100%. The addition of cystine or

TABLE 2

Mortality rate of rats receiving various levels of casein in choline-free diets.

BASAL DIET	FAT LEVEL	l-CYSTINE ADDED	NUMBER OF RATS	MORTALITY[1] RATE	AV. TIME TO DEATH	KIDNEY LESIONS	BASAL DIET	FAT LEVEL	l-CYSTINE ADDED	NUMBER OF RATS	MORTALITY[1] RATE	AV. TIME TO DEATH	KIDNEY LESIONS
		%		%	*days*	%			%		%	*days*	%
9	[3]		8	75	14	100	18	[3]	0.3	4	25	9	25
9	[3]	0.3	8	100	12	100	18	[3]	0.5	4	100	9	100
9[2]	[3]		8	87.5	14	87.5	18	[3]	1.0	4	100	9	100
9[2]	[3]	0.3	8	100	11	100	18	[3]	1.5	4	100	12	100
12	[3]		20	100	10	100	18[2]	[3]	0.3	4	75	10	100
12	[3]	0.3	4	100	9	100	18 L/2[2]	[4]	0.3	4	75	10	100
12[2]	[3]	0.3	3	100	9	100	24	[3]		4	25	9	25
12 L/2[2]	[4]	0.3	11	100	9	100	24	[3]	0.3	4	25	9	37.5
15	[3]		4	75	10	75	24 L/2	[4]	0.3	4	25	9	75
15	[3]	0.3	4	100	10	100	30	[3]	0.3	4	0		0
18	[3]		8	25	9	37.5	30 L/2	[4]	0.3	4	25	10	50

[1] Mortality rate indicates rats dying of hemorrhaged kidneys within the 3-week experimental period. Any survivors on the twenty-first day were killed and examined for kidney lesions.

[2] 20 mg of nicotinic acid was added per kilo of diet for these groups.

[3] 0.10 ml corn oil/rat/day.

[4] 15% pure lard in diet.

cystine and fat tended to increase the incidence of kidney lesions and the rate of mortality at the higher levels of casein and to decrease the survival period at the lower levels.

Mulford and Griffith ('42) have suggested that the harmful effect of cystine in choline-free diets is related to increased rate of growth. In our experiments, however, cystine added to a low-fat, low-casein diet depressed rather than increased the growth rate (table 3).

Although 24% of casein in the low-fat diet did not afford complete protection against kidney lesions, 0.38% of free dl-methionine added to a 12%-casein diet did. When the methionine was supplied, the further addition of choline was

TABLE 3

Gains of rats receiving various levels of casein, cystine, methionine and other supplements in low-fat diets for 8 weeks.

BASAL DIET	DIET CHANGE OR SPECIAL SUPPLEMENT ADDITION	NO ADDED CYSTINE			0.30% ADDED l-CYSTINE		
		Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
			gm			gm	
6		4	—1		4	—5	
6	0.38% Methionine	4	—2				
9		8	36	8.20	8	21	10.52
9	0.38% Methionine	4	28	8.60			
9	6% Yeast	4	89	4.48	4	89	4.18
12		24	59	5.44	24	32	7.53
12	0.38% Methionine	12	36	6.33			
12	0.38% Methionine without choline	12	66	4.92			
12	6% Yeast	8	147	4.22	8	157	3.69
12	6% Yeast residue[1]	4	77	5.23	4	70	5.13
12	6% Liver residue[2]	4	94	4.70	4	88	4.43
12	6% ≅ Liver extract[3]	4	108	4.90	4	132	4.13
12	Glucose for sucrose	4	98	5.65	4	69	6.40
12	Cornstarch for sucrose	12	113	4.65	12	96	4.78
15		4	111		4	77	
18		12	115	3.88	12	93	4.13
18	0.38% Methionine	4	116	3.51	4	104	3.48
18	0.38% Methionine without choline	4	126	3.54	4	122	3.60
18	6% Yeast	16	195	3.69	8	182	3.84

[1] The residue from exhaustive extraction of brewer's yeast with 51% (by wt.) alcohol.

[2] The residue from similar extraction of dried beef-liver.

[3] The 51%-alcohol extract from dried beef-liver, concentrated under diminished pressure, and added to diet at level equivalent to 6% of dried liver.

not beneficial (table 3). Likewise, 0.38% dl-methionine in a diet containing 12.5% casein and 30% lard afforded complete protection against kidney lesions in rats that made an average gain of 156 gm in 8 weeks (table 4). In contrast with this, rats

receiving 30% casein, 0.30% cystine and 15% lard showed 50% incidence of kidney damage and 25% mortality. Free methionine was clearly more effective in preventing kidney damage than methionine combined in the casein molecule. This augments the finding of Eckstein and co-workers (Tucker and Eckstein, '37; Tucker, Treadwell and Eckstein, '40; and Treadwell, Groothuis and Eckstein, '42) that the lipotropic action of free methionine was superior to that of methionine combined in casein.

Best and Ridout ('40) and Channon et al. ('43) have not agreed that the lipotropic effect of protein can be explained on the basis of methionine alone. More recent work from Best's laboratory (Beveridge et al., '44) has shown no significant difference in the lipotropic action of free methionine and methionine combined in casein provided that the essential amino acids were approximately equal. One would expect more of the methionine methyl to be available for choline synthesis when methionine is fed as the sole supplement than when it is fed along with other essential amino acids or combined with other essential amino acids in the protein molecule.

Cystine fails to supplement casein in low-fat diets

The growth of rats receiving low-fat diets containing various levels of casein, the usual crystalline vitamins, and choline or methionine as a source of transferable methyl groups, was studied next. The data are summarized in table 3. It is apparent that such diets even at the 18% casein level fall far short of producing normal growth. At the 12 and 9% casein levels growth was only slightly above one-fourth and one-sixth, respectively, of the normal rate.

The addition of cystine not only failed to increase the rate of growth but actually decreased it, particularly at the 9 and 12% levels of casein.

The addition of 6% brewer's yeast produced a remarkable response in growth. The increase was very significant at the 9 and 12% casein levels, and growth approached the normal rate at the 18% level. Liver extract also markedly increased

growth on the 12% casein diet. Moreover, yeast or liver extract counteracted the depressing effect of cystine.

The extracted residues of yeast or liver were relatively ineffective in increasing growth. When sucrose was replaced by cornstarch there was a significant improvement in growth but the rate was still abnormal.

Fat increases growth rate and alters effect of cystine

Since the diets used by Osborne and Mendel had contained substantial amounts of fat, the effect of adding 30% lard to the basal diet was studied. The casein was increased to provide the same ratio between protein and nonprotein calories as in the low-fat diets. The results are presented in table 4.

TABLE 4

Gains of rats receiving various levels of casein, cystine, methionine and other supplements in 30%-fat diets for 8 weeks.

BASAL DIET	DIET CHANGE OR SPECIAL SUPPLEMENT ADDITION	NO ADDED CYSTINE			0.30% ADDED l-CYSTINE		
		Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
			gm			*gm*	
8.3 L		3	10	17.30	4	31	6.90
8.3 L	0.38% dl-Methionine	4	40	5.77			
12.5 L		8	76	3.79	8	141	2.66
12.5 L	0.38% dl-Methionine	8	126	2.67			
12.5 L	0.38% dl-Methionine without choline	4	156	2.80			
16.5 L		12	135	2.96	8	171	2.47
16.5 L	0.38% dl-Methionine	4	183	2.28	4	176	2.27
16.5 L	0.38% dl-Methionine without choline	12	186	2.42			
25 L		16	199	2.47	16	225	2.35
25 L	0.45 dl-Methionine	4	207	2.23	4	200	2.10
25 L	0.45 dl-Methionine without choline	4	227	2.10			
25 L	6% Yeast				7	238	2.63
25 L	Cornstarch for sucrose	4	201	2.52	4	196	2.50
25 L	Butterfat for lard	4	204	2.96	4	205	2.46
25 L	Margarine fat for lard	4	204	2.86	4	204	2.49
25 L	Coconut oil for lard	4	189	3.04			
41 L		8	205	2.23	8	214	2.24

The fat addition resulted in marked improvement of growth at all levels of protein. Whereas all rats had lost weight on the low-fat, 6%-casein diet, all gained on the 30%-lard, 8.3%-casein diet. On the 25%-casein diet with 30% lard, the gains were essentially normal and about double the gain on the corresponding 18%-casein, low-fat diets when crystalline vitamins were used as the only supplements.

Another striking effect of the fat addition was that cystine additions resulted in further improvement of growth rate at low levels of casein. Moreover, brewer's yeast, which had effected marked improvement in growth on the low-fat diets, had little effect with the fat diets containing 25% casein.

Butterfat and margarine fat were comparable to lard in stimulating growth. Coconut oil was only slightly less effective; this may have been caused by a lack of essential unsaturated fat acids in the coconut oil as no other source of these dietary factors was supplied with the fat diets. The general effect of fats in increasing the rate of growth, however, was not associated with unsaturated fat acids. The corn oil fed with the low-fat diets furnished an adequate supply of linoleic acid.

Nicotinic acid or tryptophane corrects second deficiency of casein in low-fat diets

After the marked growth-stimulating effect of fat was observed and it was found that the active principle of yeast and liver was water-soluble, increased levels of thiamine, riboflavin, pyridoxine, and Ca pantothenate were tested. The additions of para-amino-benzoic acid, 2-methyl 1, 4-napthoquinone, and of biotin,[3] all of which had previously given negative results in similar diets, were rechecked. The results of these tests were negative. Nicotinic acid also had given negative results with rats in this laboratory in 1939, when added to a black-tongue-producing ration. No convincing evidence of its need by rats had appeared from other labora-

[3] Supplies of biotin were donated by the Medical Department of Merck and Co., Rahway, N. J.

tories. This vitamin fortunately was not included as a routine supplement to the diets in the early part of the investigation. Dann's ('41) report, appearing shortly after the study was initiated, seemed to prove conclusively that dietary nicotinic acid was not needed by the rat.

Since the diet used by Dann contained 25% casein, however, it was decided to try nicotinic acid as a supplement to the low-casein, low-fat diets. The results (table 5) were surprising indeed. When 20 mg of nicotinic acid per kilo of diet was added,

TABLE 5

Effect of nicotinic acid or tryptophane on gains of rats receiving various levels of casein, cystine, and fat for 8 weeks.

BASAL DIET	FAT LEVEL	l-CYSTINE ADDED	NO NICOTINIC ACID			NICOTINIC ACID ADDED		
			Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
		%		gm			gm	
9	0.10 ml corn oil/rat/day	0.1	4	22	9.70	4 [1]	63	5.19
12	0.10 ml corn oil/rat/day		4	44	5.89	3 [1]	68	4.69
12	0.10 ml corn oil/rat/day	0.3	8	27	8.07	8 [1]	104	4.22
12	0.10 ml corn oil/rat/day	0.3	4	38	7.37	4	175	4.08
12	0.10 ml corn oil/rat/day	0.3	4 [2]	171	3.70	4 [2]	172	3.83
12	0.10 ml corn oil/rat/day	0.3	4	24	8.41	4	137	3.49
12 L/2	15% lard	0.3	4	62	4.03	4	162	2.84
16.5 L	30% lard	0.3	4	166	2.38	4 [1]	179	2.39
18	0.10 ml corn oil/rat/day	0.3	8	90	4.18	12	180	3.66
18 L	30% lard	0.3	4	198	2.31	4	205	2.26
25	0.10 ml corn oil/rat/day	0.3	4	196	3.73	4	199	3.80
25 L	30% lard	0.3	4	218	2.23	4	227	2.21

[1] The diet for these 4 groups of rats contained 1 mg % of nicotinic acid; the diet for all other rats receiving nicotinic acid contained 2 mg %.

[2] 0.3% dl-tryptophane was added to the diet for these 2 groups.

improvement in growth was as striking as when brewer's yeast or liver extract was added. The effect was greatest at the 12% level of casein but was very significant even at the 18% level. With 25% of casein in the low-fat diet, growth was essentially normal and was not improved materially by nicotinic acid.

Nicotinic acid, moreover, had a similar effect to yeast and fat in permitting cystine to manifest a supplemental action at low levels of casein.

In the diet containing 30% lard and 16.5% casein, which on a protein: calorie basis was comparable to the 12%-casein, low-fat diet, nicotinic acid had a relatively slight effect on growth. When the casein was increased to 25% in the lard diet, which is comparable to the 18% of casein in the low-fat diet, there was no significant effect of nicotinic acid on growth.

In the meantime the Wisconsin workers (Krehl et al., '45) reported that tryptophane had an effect similar to that of nicotinic acid in supplementing diets having a relatively high content of corn products The addition of 0.30% dl-tryptophane to the 12%-casein diet produced about the same growth as the addition of 20 mg of nicotinic acid per kilo of diet. There was no apparent advantage to the addition of both tryptophane and nicotinic acid. These results showed definitely that the second limiting factor in the low-fat diets containing 18% or less of casein was nicotinic acid or tryptophane. Since the completion of these experiments, Krehl et al. ('46) have reported that the relationship between nicotinic acid and tryptophane is not peculiar to corn-supplemented rations but is related to the nature of the total amino acid content of the ration.

Results emphasize protein, fat, amino acid, and vitamin relationships

The results reported in this paper illustrate how the apparent nutritive value of a protein may be influenced by other dietary constituents. First in importance is the effect of

transferable methyl groups. In the absence of choline from the diet, labile-methyl groups become the first limiting factor of casein. For strains of rats having a relatively high choline requirement (Engel, '43; Copeland, '44), this limitation is disastrous in diets containing 15% or less of casein. It is significant even at levels of 24 to 30% casein, particularly if the diets contain cystine and fat.

The effect of nicotinic acid is also of major importance. It appears that there is maximum need for nicotinic acid when the diet is high in sugar and low in fat. In the absence of adequate nicotinic acid, the rat may be able to convert tryptophane to nicotinic acid to supply the metabolic requirements for this vitamin. This view is supported by the results of Rosen, Huff and Perlzweig ('46) and of Singal et al. ('46), indicating that tryptophane may be the precursor of nicotinic acid synthesis in the rat. Obviously in the low-casein diets the tryptophane supply is not adequate for this purpose and for tissue formation at the normal rate. The relatively high level of 25% casein is required to furnish sufficient tryptophane to build tissues and meet the nicotinic acid requirements of the growing rat on a nicotinic acid-free, low-fat diet. Wintrobe et al. ('45) have recently reported that pigs showed marked symptoms of nicotinic acid deficiency on a 10%-casein ration but not on a 26.1%-casein ration.

The marked growth-promoting effect of fat in the nicotinic acid-free, low-casein diets is explained on the basis of a nicotinic acid-sparing action of fat. The relatively high levels of fat required are in line with such an interpretation. The effect is nearly as striking and may be as important nutritionally as the thiamine-sparing action of fat. The higher energy value of fat diets and the possible economy of utilization of preformed fat as compared with the synthesis of fat from carbohydrate may be of some importance. The excellent growth that results when nicotinic acid is added to the low-fat diets, however, indicates that these are factors of minor importance. These results can be explained on the basis that nicotinic acid, like thiamine, functions primarily in carbohy-

drate metabolism. They also provide further information regarding the intrinsic importance of dietary fat.

A partial explanation of the anomalous effect of cystine in the low-fat, low-casein diets now becomes obvious. Cystine added to such diets cannot be utilized because of the deficiency of nicotinic acid or tryptophane. Only when this deficiency is remedied, is a deficiency of cystine demonstrable. It is not clear why the addition of cystine to the nicotinic acid-free, low-casein, low-fat diets should be harmful.

The low-casein diets used by Osborne and Mendel ('15) contained 28% protein-free milk, 25% fat, and 18 to 30% starch. These ingredients probably remedied the deficiencies of labile methyl and nicotinic acid in the casein. Under such conditions cystine (or sulfur containing amino acid) was the factor limiting growth at the 9 and 12% levels of casein.

Although methionine can be used as the source of labile methyl and tryptophane or casein at high levels as the source of nicotinic acid (certainly for rats and probably for pigs), the economy of using choline and nicotinic acid is obvious. The presence of these 2 vitamins increases the efficiency of protein utilization and thus makes relatively low-protein diets adequate for growth. That the nicotinic acid-sparing action of fat may also play an important role in practical nutrition is clearly indicated. In decreasing the need for nicotinic acid, fat may indirectly conserve ingested tryptophane and thus increase the efficiency of protein utilization. The protein required per gm of gain was significantly less in the animals receiving 30% fat than in those receiving the low-fat diets.

SUMMARY

1. The primary deficiency in diets containing 18% or less of casein was found to be labile-methyl groups unless supplementary choline or methionine was added.

2. The deficiency of labile methyl was aggravated by the addition of cystine or cystine and fat.

3. Supplementary methionine as the free amino acid was more effective than methionine combined in the casein molecule.

4. The second demonstrable deficiency in low-fat diets containing 18% or less of casein was nicotinic acid.

5. Tryptophane or a high level of casein (25%) counteracted the deficiency of nicotinic acid.

6. A high level of fat (30%) tended to counteract the deficiency of nicotinic acid. This was probably a nicotinic acid-sparing effect as the energy metabolism shifted from carbohydrate to fat and indicates that nicotinic acid, like thiamine, functions primarily in carbohydrate metabolism.

7. Only when the deficiencies of labile methyl and nicotinic acid were remedied, was a deficiency of cystine (or sulfur amino acid) demonstrable.

8. Methionine corrected both the labile methyl and the sulfur amino-acid deficiencies.

9. The possible nutritional significance of the findings is discussed.

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RELATION OF CORN PRODUCTS TO THE REQUIREMENT OF THE RAT FOR DIETARY NICOTINIC ACID[1]

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Krehl et al. ('45, '45, '45) have reported: (1) that corn products had a growth-retarding effect for rats; (2) that corn grits increased the nicotinic acid requirement of dogs; (3) that the growth-depressing effect of corn products for rats was counteracted by nicotinic acid or tryptophane or by certain proteins.

More recently Krehl et al. ('46, '46) have concluded that the relationship between nicotinic acid and tryptophane is not peculiar to corn-supplemented rations since similar effects can be demonstrated with non-corn rations which are low in both tryptophane and nicotinic acid. They report that the "deleterious action" of corn grits in a synthetic diet was prevented when fibrin, egg albumen, or soybean globulin was used in the place of casein. Moreover, the kind of carbohydrate was found to influence the extent of the "undesirable effect" of corn. These investigators suggest that the addition of corn to the diet may result in the development of unfavorable intestinal flora which may destroy nicotinic acid or that corn may contain a substance or substances which combine with nicotinic acid or in some way make it unavailable.

[1] Published with the approval of the Director of the Alabama Agricultural Experiment Station.

Any such positive harmful effects of corn would be of great nutritional importance in the South where corn bread and grits occupy such an important place in human diets. It was shown in the accompanying paper, however (Salmon, '46) that the level of casein, or tryptophane, and of fat in non-corn diets determines the need for dietary nicotinic acid by the rat. It will be shown in the following pages that this is also true in diets containing corn meal or grits and that the postulation of some kind of deleterious action peculiarly specific to corn is not supported by the data available.

METHODS AND RESULTS

The purification of the casein and the general experimental procedures were the same as in the previous investigation (Salmon, '46). The corn meal was whole white corn meal, ground on a buhr mill from locally-grown corn. The grits were the usual degerminated commercial product.

The composition of the various diets and the gains produced in rats by each diet are shown in table 1.

Corn products do not retard growth if casein level is constant

If corn had some kind of specific deleterious effect when added to a nicotinic acid-deficient diet, it would appear that the inclusion of corn in such a diet should depress the growth rate. Furthermore, one might expect that as the proportion of corn increased the depression of the growth-rate would likewise increase. The data in table 1 show, however, that the inclusion of 40% corn meal in a nicotinic acid-free basal diet, containing 9% casein, increased rather than depressed the growth rate of rats. When the corn meal was increased to 86.7%, the casein remaining at 9%, growth was substantially greater than when the diet contained only 40% corn meal. A further increase of 5% corn meal, even at the expense of the casein (corn meal 91.8%, casein 4%), resulted in a slight additional increase of growth. When casein was omitted

entirely and the diet contained only corn meal, minerals, and vitamins (no nicotinic acid included), growth was very poor but was as good as on a 9%-casein diet without corn or nicotinic acid. These data certainly fail to show any evidence of a special deleterious effect of corn meal at any level so long as the casein content of the diet is kept constant.

TABLE 1

Gains of rats receiving various levels of casein, corn products, and fat in diet[1] *for 8 weeks.*

CORN PRODUCT	CASEIN	LARD	NO NICOTINIC ACID			NICOTINIC ACID ADDED		
			Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
%	%	%		*gm*			*gm*	
	9		4	22	9.45	4[2]	63	5.09
40 Meal	9		4	55	5.96	4[2]	200	3.48
86.7 Meal	9		4	154	3.93	4	214	3.36
91.8 Meal	4		8	168	4.27	8	170	4.48
95.8 Meal			4	21	13.50	4	22	12.73
40 Meal	9		4	41	6.41	4	201	3.60
40 Meal	9		4[3]	188	3.86	4[3]	212	3.36
40 Meal	9	15	4	95	3.93	4	200	3.21
40 Meal	13.3	30	4	200	2.53	4	243	2.48
40 Grits	9		4	21	14.55	4	208	3.87
40 Meal	9		4	34	7.73	4	216	3.88
40 Grits	25		4	219	3.68	4	225	3.52
40 Grits	25	15	4	247	3.14	4	248	3.02

[1] The diets contained 4.0% of Salt 5 (Salmon, '46), 0.2% of choline chloride and sucrose to make 100%; to the diets were added the following amounts of vitamin supplements in mg/kg of diet: carotene 4, α-tocopherol 50, calciferol 0.125, i-inositol 200, thiamine 2, pyridoxine 2, riboflavin 4, Ca pantothenate 10. All diets contained 0.3% of added l-cystine except the 86.7% corn meal diet which contained only 0.1%, and the 91.8% and 95.8% corn meal diets to which no cystine was added.

[2] These 2 groups received 1 mg % of nicotinic acid in the diet; the diet for all other groups receiving nicotinic acid contained 2 mg %.

[3] These 2 groups received 300 mg % of dl-tryptophane in the diet.

The addition of nicotinic acid to the 40%-corn meal-9%-casein diet increased the gain 4 to 5-fold. The addition of nicotinic acid to the 86.7%-corn meal diet also increased growth significantly; the increase was much less but total growth was slightly greater than on the 40%-corn meal diet.

When the corn meal was increased to 91.8% and the casein decreased to 4% or when the corn meal was increased to 95.8% and the casein omitted, there was no response to the addition of nicotinic acid. Obviously some factor other than nicotinic acid was limiting growth at the 91.8% and 95.8% levels of corn meal. It appears that 86.7% of corn meal furnished enough nicotinic acid to have an appreciable effect on growth.

In the absence of nicotinic acid, growth was somewhat less when corn grits was used instead of corn meal at the 40%-level; the difference may be attributable to the difference in the nicotinic acid content of the 2 products. The response to nicotinic acid was about the same on the grits diet as on the meal diet.

The addition of 15% lard to a nicotinic acid-free diet containing 40% corn meal and 9% casein doubled the rate of growth, but the addition of nicotinic acid was still necessary for maximum growth. When 30% lard was added and the casein increased to 13.3% (to maintain the same total protein: non-protein calorie ratio as in the 9%-casein, 40%-meal diet without lard), the effect on growth was comparable to the addition of nicotinic acid. Growth was only slightly increased by the addition of nicotinic acid to the 30%-lard diet. This again shows the nicotinic acid-sparing action of fat.

When the casein level was raised to 25% in a 40%-grits diet, growth was normal and there was no response to the addition of nicotinic acid. Growth was slightly increased when this diet contained 15% lard, but the effect was much less than at the lower level of casein, because the higher level of casein supplied surplus tryptophane for conversion to nicotinic acid.

Protein and fat in diet determine dietary nicotinic acid requirement of rat

The data presented in this paper confirm the findings of Krehl et al. relating to the growth-stimulating effect of nicotinic acid or tryptophane on rats receiving diets containing 40% of corn meal or grits. They do not agree with the

hypothesis that corn in some peculiarly specific manner retards growth or alters the dietary nicotinic acid and tryptophane requirement of the growing rat. Data in table 4 of the preceding paper (Salmon, '46) have shown that, in nicotinic acid-free, low-fat diets, the casein level determines the severity of the nicotinic acid deficiency; the so-called retardation of growth can be produced by substituting 6% of sucrose for 6% of casein in a 15%-casein diet. Actually, growth was slightly increased by the inclusion of 40% corn meal in the 9%-casein, nicotinic acid-free diet and tremendously increased by the inclusion of 86.7% of corn meal. If corn had any deleterious effect, it would seem that this should have increased directly with the proportion of corn in the diet.

The most significant effect of corn is its supplementary value when added to low-casein diets containing nicotinic acid. Growth was considerably greater on a 9%-casein diet that contained 40% of meal or grits than on a 12 or even an 18%-casein diet (Salmon, '46) when the diets were supplemented with nicotinic acid. It is probable that, when the supply of nicotinic acid is ample, a mixture of casein and corn proteins is superior to casein alone. The superiority is particularly evident at moderately low levels of protein.

The growth-promoting effect of 30% of lard in diets containing 40% of corn meal without added nicotinic acid was as pronounced as was shown for casein diets in the previous paper. Thus, the author's hypothesis of a nicotinic acid-tryptophane-sparing action of fat is further supported.

Obviously the amount and nature of the protein and the amount of fat in the diet determine whether the rat has a requirement for dietary nicotinic acid. When the fat content is low and the nature or amount of the protein is such that the tryptophane supply is limited, the requirement for dietary nicotinic acid is very definite. It is apparent that when corn replaces 40% of the casein in a 15% casein diet the tryptophane content of the diet is significantly lower. In the author's opinion, the problem is a deficiency problem and there is no evidence that it is aggravated by any specific deleterious effect

of corn. The effect of fat and of tryptophane upon the nicotinic acid requirement of the rat may be likened to the effect of fat on the thiamine requirement and of methionine on the choline requirement. The supposition that the rat does not require a dietary source of nicotinic acid is false unless one presupposes a liberal supply of tryptophane or fat, or both, in the diet.

SUMMARY

1. No evidence of a specific deleterious effect of corn products in nicotinic acid-deficient rations was obtained.

2. Growth of rats was very poor on a 9%-casein diet either with or without the inclusion of 40% of corn meal or grits, when nicotinic acid was not added to the diet.

3. Growth was greater with 86.7% than with 40% corn meal in a 9%-casein diet, either with or without nicotinic acid.

4. Normal growth was produced by the addition of 1 or 2 mg % of nicotinic acid to a 9%-casein diet containing 40% corn meal or grits, or 86.7% corn meal.

5. Three hundred mg % of dl-tryptophane was only slightly less effective than 1 mg % of nicotinic acid in stimulating growth on a 40%-corn meal, 9%-casein diet.

6. A 25%-casein diet containing 40% corn grits produced normal growth without the addition of nicotinic acid.

7. The inclusion of 15% lard in a 9%-casein diet containing 40% corn meal, but no added nicotinic acid, caused a significant increase in growth. Nicotinic acid was required for normal growth on this diet.

8. When 30% lard was included in the diet containing 40% corn meal and the casein increased to 13.3%, growth was normal without the addition of nicotinic acid.

9. Fat was again found to have a marked nicotinic acid-tryptophane-sparing effect, indicating that nicotinic acid functions primarily in carbohydrate metabolism.

10. The inclusion of 40% corn meal or grits in a diet, containing nicotinic acid and 9% casein, increased growth by more than 200%, thus showing a marked supplementary value of corn in low-casein diets.

11. The conditions relating to the requirement of the rat for dietary nicotinic acid are discussed.

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THE EFFECT OF CRUDE LECITHIN ON THE COEFFICIENT OF DIGESTIBILITY AND THE RATE OF ABSORPTION OF FAT[1, 2]

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It has been recognized for years that lecithin plays an important role in fat metabolism. Lecithin is an important component of the lipid fraction of blood. It is believed that when the neutral fat is transferred from the storage depot it is first converted to a water-soluble form by phosphorylation in situ and that it is carried then in the form of lecithin to the liver. Considerable amounts of lecithin are found in the liver. For these reasons it has also been suggested that lecithin may be a form into which the fat must be transformed before it can be oxidized. Verzár and Laszt ('35a, '35b) have suggested that phosphorylation of fats must occur prior to their absorption. On removal of the adrenal cortex, it was suggested that fat absorption is slowed down because of the failure of phosphorylation. The experiments of Bavetta et al. ('41) confirm the results of Verzár and Laszt in demonstrating that a decreased absorption obtains after adrenalectomy in the rat. It was also found that this effect was due to a slowing in the absorption of fatty acids inasmuch as an accumulation of the hydrolysis products of the neutral fat occurred in the

[1] Most of these data are from a thesis presented by Virginia Augur to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

[2] Aided by a grant from the American Lecithin Co.

intestines. However, they gave no indication as to whether the depressing action was due to a failure of phosphorylation.

Frazer ('46) has given much evidence that fats may be absorbed to a considerable extent as neutral fat provided that a fine enough emulsion is produced. If this so-called partition theory of Frazer is correct, then one might expect that any agent such as lecithin which promotes the emulsification might be of aid in speeding up the absorption of fat and in increasing the digestibility of the less readily utilized fats. If, on the other hand, the lipolytic theory is correct, then one might also postulate that the presence of lecithin would increase the speed of absorption by providing the means for producing a greater emulsification which would enable a more rapid action of the lipolytic enzymes.

The results of Adlersberg and Sobotka ('43a) have indicated that both fat and vitamin A are absorbed more rapidly in man when lecithin is added to the diet. The rise in total lipids of the serum on a protein-free diet without lecithin was only 32% after 4 hours, compared with 71% in subjects on a similar diet who received lecithin along with the food. When 120,000 or 180,000 International Units (I.U.) of vitamin A were given as a component of percomorph oil, there was a rise in vitamin A content of blood plasma of only 41% when given without lecithin or an average of 212% increase if given along with lecithin. In addition, lecithin has an effect on the level of blood cholesterol. It was demonstrated by Adlersberg and Sobotka ('43b) that the prolonged administration of lecithin brought about a slight decrease in serum cholesterol, although it returned to high levels after the administration of lecithin was stopped. Similar results are recorded by Steiner and Domanski ('44), although the latter investigators found that the high levels of blood cholesterol returned after 4 or 5 weeks in spite of continued lecithin feeding. Urbach ('46), however, was able to maintain persistently low levels of blood cholesterol when a diet low in carbohydrate and fat was given along with the lecithin. The results of Adlersberg were also confirmed by Gross and Kesten ('43). Slanetz and Scharf

('43, '44) have reported that some unknown factor is necessary for the utilization of vitamin A in the rat which is present in crude lecithin and which is not vitamin E.

The total availability of the fat (i.e., the coefficient of digestibility) as well as the rate of absorption are both important in evaluating the nutritive value of fat. Although the coefficient of digestibility of poorly utilized fats and their rate of absorption are roughly parallel, differences in rate of absorption may occur in fats which are practically completely digestible. Whereas the coefficient of digestibility may be obtained by calculation from the fat ingested and that excreted in the feces, the rate of absorption can only be determined by short term tests where the actual speed of removal of the lipid from the small intestine is ascertained.

Because most of the work which has been done on the effect of lecithin on the absorption of fat has been indirect rather than direct, the present experiments were undertaken to determine whether an increased rate of absorption occurs with fats when mixed with lecithin and also whether any improved digestibility of fats obtains when additional lecithin is added.

EXPERIMENTAL

The effect of lecithin on digestibility was demonstrated by feeding rats on diets containing approximately 15% fats of high melting point with or without added lecithin. The animals were allowed a 2-day period for orientation and the feces were then collected for the following 8 days. Food consumption was recorded during this period and the diets were allowed ad libitum. At the conclusion of the digestibility experiment, the animals were fasted for 2 days after which the effect of lecithin on the rate of absorption was determined for 3- and 6-hour periods. The same fats fed on the digestibility experiments were used in the absorption tests. The diets employed are recorded in table 1.

The combined feces of the 8-day period were dried under a vacuum to constant weight, ground with a mortar and pestle and the neutral fat extracted on a Soxhlet extractor with

diethyl ether. The residue was again dried, mixed with a minimum amount of 50% sulfuric acid and the acid paste reextracted with ether. The neutral fat and fatty acid obtained were dried under a vacuum at 60°C. to constant weight. It was demonstrated that no acid could be extracted by diethyl ether from sulfuric acid solution and it was also found that the material extracted from dried feces after acidification was acid in reaction and possessed the properties of a fatty acid.

TABLE 1

Composition of diets used in digestibility studies.

COMPONENTS OF DIET	DIET 1	DIET 2	DIET 3
	%	%	%
Commercial casein	18	18	18
Glucose	56	53	71
Hydrogenated cottonseed oil	15	15	0
Salt mixture [1]	7	7	7
Yeast [2]	1	1	1
Liver extract [3]	3	3	3
Crude Soya Lecithin [4]	0	3	0

[1] Osborne-Mendel ('19).

[2] Anheuser-Busch, Strain G.

[3] Wilson and Co., 1 : 20 concentrate.

[4] Prepared by The American Lecithin Co.

The calculations for digestibility were made in the usual way, correction being made for the metabolic fat. The correction for metabolic fat was determined in experiments where the animals were fed on Diet 3 (table 1) in which the fat has been replaced by glucose. The 10 rats used in this determination weighed on an average, 209 gm, consumed a total of 94.5 gm of food, and excreted stools which had an average dried weight of 5.35 gm. The feces contained a total of 181 mg of fat as neutral fat, 87.5 mg as soap which makes a total of 268 mg or 50.5 mg per gm of dried stool. The latter figure was used in the subsequent calculations. The coefficient of digestibility was expressed as the per cent of the ingested fat which had been utilized. The amount of digested fat was

determined by subtracting the corrected excretion value from the original weight of ingested fat.

The absorption tests were carried out by the usual procedure as described in the paper of Deuel, Hallman and Leonard ('40). The fat was fed at a level of 300 mg per 100 cm^3 of body surface based on fasted weight. Surface area was estimated by the formula of Lee ('29). In some preliminary experiments where the effect of lecithin on preventing diarrhea was determined after giving large doses of cottonseed oil, the fats were fed at approximately 1.5 or 2 times the usual level. The experiments were carried out on a commercial sample of cottonseed oil [3] and on 3 samples of cottonseed oil hydrogenated to melting points of 46°, 54°, and 65°C.[4] The crude lecithin was added in the absorption and diarrhea tests in proportions of one-sixth or one-fifth, respectively. According to the manufacturer, the crude lecithin contains 30 to 40% of soybean oil and about 60 to 70% of mixed phosphatides of lecithin, cephalin and lipositol. Adult female rats were used throughout the experiment except in one series of tests on susceptibility to diarrhea and were from the University of Southern California colony.

RESULTS

In a series of preliminary experiments, tests were made to determine whether a greater amount of fat could be digested without the onset of diarrhea if lecithin was added to the diet. A summary of these experiments is recorded in table 2.

When either cottonseed oil or hydrogenated cottonseed oil was fed at several levels, the number of rats which developed diarrhea was invariably higher in the group where no lecithin was added. In a grand total of 50 experiments where fat was fed without lecithin, a total number of 20 cases of diarrhea were noted which is an incidence of 40%. Only 4 rats of the 40 which received lecithin with the fat developed a diarrhea,

[3] Wesson Oil.

[4] These fats were kindly prepared for us by Dr. K. Mattil of Swift and Co.

TABLE 2

The incidence of diarrhea in rats fed several fats with or without added lecithin after a previous 48-hour fast period.

FAT FED	LENGTH OF PERIOD	FAT ALONE				FAT PLUS LECITHIN			
		Sex	Dose	No. of tests	No. with diarrhea	Sex	Dose	No. of tests	No. with diarrhea
	hours		*mg/100 cm²*				*mg/100 cm²*		
Cottonseed oil	3	F	328	3	1				
	3	F	410	4	3	F	410	7	0
	2	F	410	23	5	F	410	16	1
	2	M	615	10	4	M	615	10	2
Hydrogenated cottonseed oil	6	F	337	10	7	F	374	7	1

TABLE 3

Summary table of digestibility of 3 samples of cottonseed oil hydrogenated to different degrees when fed in a diet without lecithin (Diet 1) or with lecithin (Diet 2).

DATA SUMMARIZED	FAT 1 (46°C.)		FAT 2 (54°C.)		FAT 3 (65°C.)	
	Diet 1	Diet 2	Diet 1	Diet 2	Diet 1	Diet 2
Number of rats	10	10	9	10	10	10
Average weight of rats, gm	191	157	173	181	241	243
Average gain, gm	6.5	12.3	10.3	8.0	0.9	—1.4
Fat in diet, %	16.6	20.2	15.7	20.7	17.6	18.4
Average food eaten, gm	88	86	82	83	90	91
Average fat ingested, gm	14.9	17.5	12.3	17.2	15.7	16.6
Average weight of dried stools, gm	7.8	6.7	9.1	8.5	17.7	15.2
Average fat excreted						
As neutral fat and fatty acid, gm	0.73	0.62	0.62	0.79	6.31	4.83
As soaps, gm	2.09	1.86	3.80	2.63	6.93	5.33
Total, corrected [1]	2.39	2.09	3.92	2.95	12.25	9.32
Coefficient of digestibility [2]	83.8 ± 1.4	87.9 ± 0.7	68.7 ± 2.7	82.8 ± 1.4	24.0 ± 2.6	44.2 ± 2.0
M.D. : S.E.M.D. [3]		2.66		4.71		6.05

[1] Corrected for metabolic fat by multiplying weight of dried stools by 50.5 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[3] Mean Difference : Standard Error of Mean Difference of results on Diet 1 compared with those on Diet 2. When this value exceeds 3, the results are considered significant.

which equals only 10%. In the cottonseed oil tests, the experiments with and without lecithin were carried out simultaneously. In the case of the hydrogenated cottonseed oil, the experiments on lecithin were carried out several days later on the 7 rats which had developed diarrhea when cottonseed oil alone was fed. The data on the digestibility of various samples of cottonseed oil are summarized in table 3.

The addition of lecithin considerably increased the digestibility of the cottonseed oil, particularly in the case of the

TABLE 4

Summary table of absorption rates of cottonseed oil, of 2 samples of hydrogenated cottonseed oil when fed (A) without or (B) with lecithin.

FAT (A) WITHOUT OR (B) WITH LECITHIN	NO. OF TESTS	DURATION	AVERAGE RAT WEIGHT	AVERAGE FAT IN MG				M.D. : S.E.M.D.[3]
				Fed	Recovered [1]	Absorbed		
						Total	Per 100 cm² per hr.[2]	
				Cottonseed oil				
		hrs.	*gm*					
A	11	2	135	911	693	238	47.8 ± 2.3	
B	14	2	132	908	635	292	61.8 ± 3.7	3.21
A	6	3	112	705	488	237	38.5	
B	8	3	121	835	460	395	59.9	
				Hydrogenated cottonseed oil (m.p. 46°C.)				
A	10	3	200	864	601	293	26.5 ± 1.8	
B	10	3	204	925	476	479	48.9 ± 3.0	3.56
A	11	6	169	775	403	402	24.7 ± 2.0	
B	9	3	207	946	323	643	36.7 ± 2.2	4.12
				Hydrogenated cottonseed oil (m.p. 54°C.)				
A	9	3	197	822	674	178	18.0 ± 1.8	
B	8	3	189	837	587	280	30.1 ± 2.8	3.58
A	9	6	197	836	709	157	8.5 ± 0.9	
B	9	6	198	870	519	381	21.8 ± 1.5	7.65

[1] Does not include the correction for lipid content of fasting rats. The value used for the cottonseed oil tests was 20 mg, and for the hydrogenated cottonseed oil tests, 30 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[3] Mean Difference : Standard Error of Mean Difference of results on "A" compared with "B." When this value exceeds 3, the results are considered significant.

highest melting sample. The low digestibility of the fat melting at 63°C. is not to be ascribed to a failure of lipolysis but to the inability to absorb the stearate so formed. We have no evidence from our tests on how large a proportion of the material which was ether-soluble before acidification was neutral fat and how much represented the free fatty acid. The total stool weight was slightly lower in all cases with the lecithin-fed rats.

The results of the absorption tests with cottonseed and hydrogenated cottonseed oils are recorded in table 4.

DISCUSSION

There would seem to be little doubt that the addition of a crude lecithin preparation to fat lessens the susceptibility to diarrhea in rats. This effect might be the resultant of a depressing action of some components of the crude lecithin on gastric or intestinal motility or secondly because it causes a more rapid rate of absorption whereby the quantity of fat present in the gut is sufficiently reduced to lessen the chance of an ensuing diarrhea.

The present experiments indicate that the above effect is probably to be traced to an acceleration in the absorption rate. In the case of cottonseed oil the rate of increase in absorption is 29% at 2 hours and 56% at 3 hours. With the hydrogenated fat melting at 46°C., the results are equally striking, being 85 and 49% at 3 and 6 hours, respectively. The increased absorption rates for the higher melting fat (54°C.) containing lecithin are 67 and 156%, respectively, for the corresponding periods.

There are several possible explanations as to why crude lecithin may increase the absorption rate. In the first place, it might be suggested that lecithin is preferentially absorbed from the fat-lecithin mixture at a faster rate than the balance of the fat. It is difficult to see how this could occur from the lecithin-fat mixtures which are entirely homogenous. Moreover, if one assumes a 100% absorption of the lecithin in all 4 series of tests with the hydrogenated cottonseed oil samples,

there still would be an additional absorption of the neutral fat in the lecithin groups over those experiments where lecithin was not administered. In the cottonseed oil tests, the increase is approximately equivalent to the lecithin fed.

Secondly, it may be argued that the lecithin can not be recovered from the gut by the flushing procedure with diethyl ether. In tests where the recovery of known amounts from the gastro-intestinal tract was tested by removing the gut immediately after the fat had been discharged into it, it was found that the average recovery after cottonseed oil was 95.4 and after cottonseed oil-lecithin mixture, 92.9%.

Thirdly, it is possible that lecithin promotes absorption by increasing the speed and degree of emulsification. Such an effect could be explained either by an increased rate of absorption of unhydrolyzed triglyceride due to increased emulsification (according to the Partition Hypothesis of Frazer), or that an increased speed of the action of the lipolytic enzymes is possible because of the greater surface for their action.

The action of lecithin in increasing digestibility must be related to its speeding up of absorption. With the large amount of fat available at one time in the intestine, it is impossible for it to be removed from the gut at the very slow rate that absorption normally obtains before it has passed beyond the absorptive portion of the gastro-intestinal tract. Anything which will increase the rate of absorption during the interval that it is in the area where it can be absorbed will obviously improve the digestibility.

SUMMARY

The addition of lecithin to cottonseed oil or to a hydrogenated cottonseed oil markedly lowers the susceptibility to diarrhea caused by a large dose of these fats to rats.

Fats containing one-sixth or one-fifth crude lecithin are absorbed more rapidly than a similar fat without any added phosphatide.

It was found that hydrogenated cottonseed oil melting at 63°C. had a digestibility of 24 in the rat; that melting at

54°C. was digested to the extent of 69% while that with a melting point of 46°C. had a digestibility coefficient of 84. These were increased by the addition of lecithin to 44, 83 and 88%, respectively.

A considerably larger portion of the lipid in all cases was excreted as soaps than as neutral fat plus fatty acids.

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A COMPARISON OF THE COEFFICIENT OF DIGESTIBILITY AND THE RATE OF ABSORPTION OF SEVERAL NATURAL AND ARTIFICIAL FATS AS INFLUENCED BY MELTING POINT [1,2]

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Fats and oils offer an excellent source of energy. Nearly all of the natural fats have been found to be from 93 to 98% digestible (Langworthy, '23). The only exceptions found in the case of man are those with high melting points and those which contain irritants such as castor and croton oil.

It has been suggested that an inverse relationship exists between the coefficient of digestibility and the melting point of the fat (Holmes and Deuel, '21). The critical temperature above which there is a marked decrease in digestibility in man appears to be about 50°C. Thus, it was found that mutton fat (Langworthy and Holmes, '15) and deer fat (Deuel and Holmes, '22) are digested to an extent of only 88 and 81.7%, respectively. Hydrogenated fats (Holmes and Deuel, '21) and blended hydrogenated fats (Deuel and Holmes, '22) also have a lower digestibility in man when the melting point exceeds 50°C. More recently it has been stated by Mattil

[1] The data are from a thesis presented by Mary E. Crockett to the Graduate School of The University of Southern California in partial fulfillmen of the requirements for the degree of Master of Science.

[2] Aided by a grant from Swift and Co.

and Higgins ('45) that the stearate content rather than melting point is the prime consideration in determining the completeness of digestibility. Inasmuch as the higher melting points of fat are usually associated with an increased proportion of tristearin, it is difficult to study these variables separately.

The nutritive value of fat can also be established by determining its rate of absorption from the gastro-intestinal tract. It is possible that a fat which is almost completely digested over a given length of time, might nevertheless have a slow rate of absorption through the intestinal wall. There are very little data at present on the rate of absorption of fats. Steenbock and others ('36) found a significant difference between the absorption rate of butter fat and coconut oil, whereas Deuel et al. ('40) found no consistent differences. This was probably due to the units employed for expressing rate of absorption. Deuel et al. ('40) found that uniform results were obtained when comparison of absorption rate was made on the basis of body surface area.

The present experiments were designed to compare the digestibility of several of the commercially hydrogenated products in the rat with other natural fats and to determine whether a satisfactory digestibility occurs with a blended hydrogenated fat, namely bland lard.[3] The tests were made on rats since the digestibility of fats in this animal is particularly effected by a high melting point. The tests were also designed to determine whether the rate of absorption is altered before the digestibility. A further discussion of the importance of terms "digestibility" and "absorption" as used in this laboratory is given in the earlier paper (Augur et al., '47).

EXPERIMENTAL AND RESULTS

Digestibility and absorption experiments were carried out on a commercial margarine made from hydrogenated vegetable

[3] Bland lard is a blended hydrogenated fat composed of a mixture of lard hydrogenated to a high melting point and deodorized lard containing an antioxidant.

TABLE 1

Summary table of digestibility of hydrogenated fats when fed to female rats in a diet at a level of 15%.

DATA SUMMARIZED	MARGARINE	CRISCO	BLAND LARD	PRIME STEAM LARD	HYDROGENATED LARD	HYDROGENATED LARD
Melting point, °C.	34	43	48	37	55	61
Number of rats	20	20	18	16	18	20
Average weight of rats, gm	214	207	208	229	244	223
Average gain, gm	—1	+5	+1	+2	—4	—6
Average food eaten, gm	72	82	85	81	90	88
Average fat ingested, gm	10.7	12.3	12.8	12.2	13.5	13.2
Average weight of stools, gm	4.40	5.50	5.86	5.36	10.0	15.0
Average fat excreted						
As neutral fat and fatty acids, gm	0.24	0.23	0.34	0.21	0.58	2.21
As soaps, gm	0.30	0.38	0.68	0.48	4.90	8.89
Average total fat excreted (corrected), gm [1]	0.31	0.33	0.72	0.39	4.94	10.34
Coefficient of digestibility [2]	97.0 ±0.4	97.3 ±0.3	94.3 ±1.8	96.6 ±1.4	63.2 ±1.2	21.0 ±2.6

[1] Corrected for metabolic fat by multiplying weight of dried stools by 50.5 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

oils, Crisco, bland lard,[4] prime steam lard,[4] and lards hydrogenated to 55° and 61°C.[4] The diets and the methods of analyses were entirely similar to those employed in the previous study (Augur, Rollman and Deuel, '47). The tests were made on adult female rats from the University of Southern California colony.

[4] These fats were kindly furnished us by Swift and Co. The samples of hydrogenated lard were prepared for us by Dr. K. Mattil of that organization. The authors wish to thank Mr. J. H. Luckmann of The Best Foods, Inc., for checking the melting points.

TABLE 2

Summary table of absorption rate of 5 samples of fat.

DATA SUMMARIZED	MARGARINE		CRISCO		BLAND LARD		PRIME STEAM LARD		HYDROGENATED LARD — 55°C.	
	3-hour tests	6-hour tests	3-hour tests	6-hour tests	3-hour tests	6-hour tests	3-hour tests	6-hour tests	3-hour tests	6-hour tests
No. of rats	10	10	10	10	10	10	10	10	12	10
Average fasting weight, gm	198	202	198	200	177	185	227	200	209	216
Average fat fed, mg	875	889	875	874	809	835	950	883	906	898
Average fat recovered, mg [1]	519	240	516	217	494	254	590	166	688	482
Average fat absorbed, mg, total	356	649	358	657	315	581	360	717	218	416
Per 100 cm^2/hr.[2]	38.7 ±1.5	34.7 ±1.0	37.1 ±2.0	34.3 ±0.6	34.5 ±1.7	31.4 ±1.6	35.0 ±1.9	38.3 ±2.02	20.7 ±3.5	21.6 ±3.5

[1] Correction for lipid content of intestine of 2-day fasted rats. 36.3 mg was subtracted from the amount recovered for each test.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

Digestibility studies

Sixteen to 20 digestibility experiments were made on each fat. The average results are summarized in table 1.

There are no significant differences in the coefficient of digestibility between the fats with melting points less than 55°C. The range is from 94.3% for bland lard to 97.3% for Crisco. The hydrogenated lards with a melting point of 55°C. and above show a marked decrease in digestibility. The weight of the stools increased with the increase in melting point. The lack of digestibility of the hydrogenated lards is not due to a failure of lipolysis but to the inability to absorb the palmitate and stearate formed from this action.

Absorption tests

The average results of the absorption studies are given in table 2. The rats were fed the same fats as used in the digestibility studies. No tests were possible on the hydrogenated lard melting at 61°C. since the temperature of the liquid phase was high enough to cause injury. No accurate method of feeding it as an emulsion was found whereby a quantity comparable to that of the other fats could be fed.

DISCUSSION

Margarine, Crisco, prime steam lard and bland lard have all been shown to be practically completely digestible in the rat and to compare favorably with natural fats and oils in which the melting point is below 50°C. The value for margarine is identical with that recently reported in human subjects (Deuel, '46) and that for prime steam lard is almost identical with that found by Langworthy and Holmes ('15) in man. As would be expected, the hydrogenated lard samples melting at 55°C. and 61°C. were much less digestible than other fats. In experiments on man, practically no decrease in digestibility obtained with hydrogenated vegetable fats melting at 50°C. (Holmes and Deuel, '21) but with rats only a 63% utilization occurred. In fact, with hydrogenated fats

melting as high as 52.4°C. (Deuel and Holmes, '22), the lowest digestibility found with man was 79.

The discrepancy between the results on man and the rat may be a species difference. Herbivora digest high melting fats much less satisfactorily than omnivora or carnivora (McCay and Paul, '38) but are able to utilize castor oil as well as other fats (Paul and McCay, '42). On the other hand, the variation may be due to the technic employed. In the tests on human subjects, the feces were extracted with diethyl ether without acidification whereby only the neutral fat and free fatty acids would be recovered. Any fatty acid in the form of soap would be lost. In the present tests after extraction of the neutral fat and fatty acids, the residue was acidified and reextracted with diethyl ether whereby the fatty acids formed from the soaps would then be extracted. While the quantity present as soap is very small in the stools after such completely digestible fats as margarine, Crisco, and the two lard samples were fed, it amounted to 4 to 6 times the quantity of undigested neutral fat plus free fatty acid when the hydrogenated lards were fed. With the exception of Hoagland and Snider ('43), the previous investigators have not generally taken the soaps into account. It is possible that if the tests on humans are made using a similar technic, a considerable amount of soap might be found with the high melting fats and a greatly decreased digestibility would be demonstrated.

The failure in absorption of the high melting fats cannot be due to the inadequacy of lipolysis. The fatty acids set free far exceed that required to produce a satisfactory emulsion (when combined with monoglyceride and bile salts) according to the Particulate Hypothesis of Frazer ('46). It is possible that emulsification is incomplete with the high melting fats or that the fat particles may be too large to be absorbed through the intestinal mucosa. The improved utilization of hydrogenated cottonseed fat when lecithin is mixed with it is demonstrated by Augur et al. ('47) may be the result of improved emulsification with increased absorption of neutral fat.

The high digestibility of the bland lard which contains a portion of the completely hydrogenated lard indicates that the digestibility of high melting fat is considerably increased when fed as a component of a blended fat having a melting point in the physiological range. It is similar to the results obtained on human subjects with blended fats (Deuel and Holmes, '22) although no account was taken in these earlier tests of the possible excretion of fat as soaps.

There is a decrease in rate of absorption of the hydrogenated lard melting at 55°C. compared with the lower melting fats which is proportional to the decrease in digestibility. Thus the decrease in absorption rate for the hydrogenated lard (m.p. 55°C.) compared with prime steam lard was 41% while the digestibility was lowered by 35%.

There was no diarrhea resulting from the feeding of rats diets containing 15% of the different fats over a 10-day period. Also no diarrhea occurred in the absorption tests. In the digestibility tests, the rats appeared to be normal at the end of the experiment and no upset in nutritional condition was noted.

SUMMARY

1. The digestibility of various fats has been studied. The digestibility coefficients obtained were margarine (m.p. 34°C.), 97.0; Crisco (m.p. 43°C.) 97.3; prime steam lard (m.p. 37°C.), 96.6; bland lard (m.p. 48°C.), 94.3; hydrogenated lard (m.p. 55°C.), 63.2; and hydrogenated lard (m.p. 61°C.), 21.0.

2. There was no evidence that any fat produced any abnormal physiological effect, such as diarrhea.

3. The rates of absorption for 3 hours, expressed in mg per 100 cm^2 per hour, were: margarine, 37.7; Crisco, 37.1; prime steam lard, 35.0; bland lard, 34.5; hydrogenated lard (m.p. 55°C.), 21.6. The values obtained for 6 hours were: margarine, 34.7; Crisco, 34.3; prime steam lard, 38.3; bland lard, 31.4; hydrogenated lard (m.p. 55°C.), 21.6.

4. A large proportion of the undigested fat in the tests with the hydrogenated lards was excreted in the form of soaps.

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THE USE OF ALBINO MICE TO DETERMINE THE UTILIZATION OF THE CALCIUM OF DEHYDRATED CARROTS AND CABBAGE

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The literature on the availability of calcium in vegetables has been reviewed briefly by Fincke and Sherman ('35) and Fincke ('41). Most of this work has been carried out either by balance studies on human beings or by deposition experiments using weanling albino rats with the comparison of the utilization of a given foodstuff to that of milk.

The availability of dietary calcium, in foods other than dairy products, assumes a far greater importance in those regions with a negligible dairy industry such as the Far East where the authors have been for several years. One of us, Bendaña-Brown, was studying the availability of calcium in various Philippine foodstuffs in Manila from 1938 until the capture of the city in January, 1942. Preliminary results by Bendaña-Brown and Geronimo [1] ('41) showed that the calcium of pechay, a Philippine vegetable similar to Chinese or celery cabbage with a high calcium content (about 1% when dried), had an availability of 75 to 90% of that of dried skim milk, and that soy bean curd cheese (tokua) had an availability of about 80 to 90% of that of dried skim milk when tested by the rat deposition method of Fincke and Sherman ('35) at a dietary calcium level of 0.26%. Kung, Yeh and Adolph ('38), feeding rats at a dietary level of 0.10% cal-

[1] Killed in the reoccupation of Manila in February, 1945.

cium, found 95 to 99% utilization of the calcium of celery cabbage while Kao, Conner and Sherman ('38) found a value of about 80% when half of the calcium of the milk diet was replaced by the calcium of Chinese cabbage at a dietary calcium level of 0.26 to 0.28%. Adolph and Chen ('32) working with 3 Chinese adults in balance experiments, found that soy bean curd was approximately equal to milk as a source of calcium. Potgieter ('40a, '40b), in Hawaii, found that a diet in which 97% of the calcium was furnished by taro was well utilized by both women and growing rats. Potgieter ('40a), using rats in paired feeding experiments with $Ca(H_2PO_4)_2 \cdot H_2O$ as a control and feeding at levels of dietary calcium below 0.10% with taro furnishing 97% of the dietary calcium, found that 82% of the taro calcium and 88% of the inorganic salt calcium was utilized. The same author ('40b), testing 1 Caucasian woman and 1 Japanese woman, found that the latter would maintain calcium balance on a lower calcium level than the former. However, Steggerda and Mitchell ('46) have shown a considerable normal variation (23%) in the calcium metabolism and requirements of normal Caucasian adults. Lunde and Lie ('40) and Basu, De and Basak ('42) have found that the calcium of the bones of small fish possesses about the same availability as that of milk. MacDonald and Bose ('42), have found that egg shells are beneficial to rats fed on a diet similar to that eaten by the poor Indian population. In the Santo Tomas Internment Camp in Manila, during the Japanese occupation, a number of us supplemented our meager rice and corn diet with pulverized egg shells partially dissolved in vinegar. Basu, Basak and De ('42) found that when 6 betel nut leaves were chewed with lime (a portion of a betel nut plus the rolled leaves and a portion of slaked lime and often some tobacco are all chewed together producing a blood red sputum which stains the lips and teeth), calcium equivalent to 10 oz. of milk was available and that the calcium so obtained was well retained and utilized. In the Philippines this habit is widely practiced and considered to be of particular value during

pregnancy. Kelly ('43) has found for rats utilization values of 80% to 86% for milk, 37% for beet greens, 70% for Swede turnips, 80% for Savoy cabbage, and 76% for parsnips. Eighty-six per cent of the calcium intake in these experiments was derived from the test food which was fed fresh, raw, and uncooked at a suboptimal level. Breiter, Mills, Rutherford, Armstrong and Outhouse ('42), in balance experiments with 4 men and 3 women, found utilization values of from 0.0 to 35.5% (average 13.4%) for the calcium from carrots. These same subjects had previously been used by Breiter, Mills, Dwight, McKay, Armstrong and Outhouse ('41) for similar experiments with milk where the values obtained were appreciably higher in 5 of the 7 cases.

The authors felt that a more rapid method of biological determination of the relative utilization of calcium of various sources would be of value. In an effort to bring this about, the following work with albino mice was undertaken. We wish to express our sincere appreciation to Prof. H. B. Lewis and his staff for facilities and advice during our work which was terminated by the necessity of an early return to Manila.

EXPERIMENTAL

The method used is based on that introduced by Fincke and Sherman ('35) for rats. Albino mice were weaned at 18 to 21 days of age and placed on the various experimental diets, the composition of which is given in table 1. The diets for a given experiment were all prepared at the beginning of the experimental period and a sample of each was ashed and analyzed for calcium.

Litter mates of the same sex were used in the comparison of the various diets. The animals of each litter group were placed on the diets as indicated in the tables and one or more of each litter were killed at the beginning of the experiment for calcium analysis as a base line control of the calcium content of that litter at the beginning of the feeding period. The median weight mouse in each litter was used for the base line control and the one nearest to it in weight served as the

experimental control on the basic 20% dried skim milk diet (diet M-2a). The mice were kept in individual metabolism cages with raised screen bottoms. Records were kept of the food consumed. The experiments were continued until the animals doubled or tripled their initial weights which was in general at about 40 days of age or after a feeding period of 18 to 21 days. They were then killed with chloroform

TABLE 1

Percentage composition and calcium content of the experimental diets.[1]

DIET NO.	GROUND POLISHED RICE	DRIED SKIM MILK	CORN STARCH	DEHYDRATED CARROTS	DEHYDRATED CABBAGE	CALCIUM CONTENT
						%
M-2	65	20	1			0.279
M-3[2]	65	15	5.5			0.281
M-4[3]	65	10	10			0.280
M-5	60	25	1			0.343
M-6[3]	65	20	0.5			0.345
M-2a	65	20				0.280
Cab-1	47	10			28	0.276
Cab-2	52	13			20	0.281
Cab-3	57	16			12	0.282
C-3	34	13		38		0.278
M-3b	70	15				0.222
M-4b	75	10				0.172
M-5a	60	25				0.343

[1] In addition to the constituents listed, all diets contained 1.5% sodium chloride (C.P.), 0.5% of dried beef liver (prepared in this laboratory), 2% of dried yeast powder (Mead Johnson), 2% of cod liver oil and 9% of corn oil (8% in diets M-2 to M-6, inclusive).

[2] 0.5% of calcium lactate added.

[3] 1.0% of calcium lactate added.

and the bodies, minus the digestive tract, were ashed in platinum dishes and analyzed for calcium by the A.O.A.C. volumetric method ('40) with minor modifications.

In order to minimize the labor involved and to obtain the most consistent results, it was found advisable to provide a 5-oz. wide mouth bottle for each experimental animal and to weigh into this at the beginning of the experiment the food

for the entire period, usually about 60 gm. These bottles were kept in the refrigerator and food was transferred to the food cups daily and mixed with water to a paste which almost entirely eliminated scattering. With a little practice, it was possible to adjust the amounts of food dispensed daily so that little or none remained and the over-all labor saved was considerable. Since the only weighings of food necessary were those at the beginning and the end of the experiment, it was possible to feed 25 to 30 experimental animals in 30 to 40 minutes daily.

The cabbage and carrots used were dehydrated vegetables obtained for us by Prof. H. B. Lewis through the courtesy of Major George Berryman of the Chicago Medical Nutrition Laboratory of the Armed Service Forces and Colonel John B. Youmans. Each vegetable and the mixed diets were analyzed for calcium, all materials being finely ground and mixed thoroughly before sampling for analysis.

DISCUSSION AND RESULTS

In planning our diets (table 1), it seemed advisable to use diets which could be easily reproduced either in this country or in the Philippines for future work. This made it desirable to replace whole wheat with polished rice. Rice had the further advantage of being very low in calcium (0.009%) so that the amount of rice could be varied without materially affecting the calcium content of the diet. The previous work of Fincke and Sherman ('35) shows that, when rather large amounts of dried vegetables are used, the protein content of the experimental diets often exceeds that of the skim milk control diet. The replacement of the corn starch of the Fincke and Sherman diet with rice of 8% protein content tends to give control and experimental diets of a more nearly equal protein content.

As there were no available data on mice, an attempt was made in the first experiments to determine whether or not the procedure of feeding a 20% dried skim milk diet as a control and replacing one-half of the milk with an equivalent

amount of calcium from the source to be tested, as the Sherman group have done with rats, was applicable to this other species. It was felt that a study of the utilization of calcium at various levels of milk intake, similar to those to be used in the experimental work and balancing the calcium levels with a readily available source such as calcium lactate, would be a valuable test. Five litters of albino mice were used, 1 animal of each litter was killed as a measure of the calcium content of the mice in its respective litter and 1 mouse of

TABLE 2

The effect of varying levels of intake of calcium and dried skim milk on the utilization of dietary calcium in weanling male albino mice.[1]

DIETS			FOOD INTAKE	GAIN IN WEIGHT		CALCIUM	
No.	Dried skim milk	Calcium		Total	Per gm of food	Increment	Utilization factor
	%	%	*gm*	*gm*	*gm*	*mg*	%
M-2	20	0.279	49.7	10.2	0.21	83	61 ± 3.1 [2]
M-3	15 [3]	0.281	51.3	10.6	0.20	83	58 ± 2.2
M-4	10 [4]	0.280	59.3 [5]	10.2 [5]	0.17 [5]	88	54 ± 2.0
M-5	25	0.343	50.1	9.2	0.19	86	51 ± 3.1
M-6	20 [3]	0.345	48.9	10.6	0.22	86	52 ± 2.5

[1] The values for diets M-2, M-3 and M-4 represent averages of 5 animals; those for diets M-5 and M-6, averages of 4 animals.

[2] Probable error of the mean.

[3] 0.5% of calcium lactate added.

[4] 1.0% of calcium lactate added.

[5] Data from 1 animal (G-3) that ate much less than the others and gained only 2.3 gm are excluded.

each litter was placed on each diet. Dried skim milk, at levels of 10, 15, 20 and 25% was fed and the calcium level of the diets containing 10 and 15% was balanced at those of 20 and 25% dried skim milk by adding the necessary amounts of calcium lactate as shown in table 2. The animals on the 10% skim milk plus calcium lactate made similar gains to those on the 20% dried skim milk but did not make quite as efficient use either of the total food or of the ingested calcium, showing an average gain per gm of food eaten of 0.17 gm and a utiliza-

tion of 54 ± 2.0% as compared with 0.21 gm and 61 ± 3.1%, respectively, for the mice on the 20% dried skim milk diet. This difference in utilization was only of a very slight statistical significance. We, therefore, felt justified in the use of 20 and 10% levels of dried skim milk, the levels commonly used in rat experiments of this type. It is desired to call attention to the slightly poorer utilization of calcium at the higher level of intake, 51 ± 3.1% at 0.343% calcium intake as against 61 ± 3.1% at the 0.279% level. While these differences are slight, it is believed that they are significant as is further shown in table 3.

TABLE 3

The utilization of dietary calcium by weanling male albino mice at various intake levels.[1]

DIETS			FOOD INTAKE	GAIN IN WEIGHT		CALCIUM	
No.	Dried skim milk	Calcium		Total	Per gm of food	Increment	Utilization factor
	%	%	*gm*	*gm*	*gm*	*mg*	%
M-5a	25	0.343	40.2	8.4	0.21	61	45 ± 1.1[2]
M-2a	20	0.280	43.6	10.4	0.24	80	66 ± 1.8
M-3b	15	0.222	42.9	9.7	0.23	74	77 ± 2.0
M-4b	10	0.172	41.4	9.3	0.22	61	86 ± 3.7

[1] All values are averages of results with 5 mice.

[2] Probable error of the mean.

The utilization of the calcium of dehydrated carrots and cabbage was tested at several levels of intake. In a preliminary experiment (not shown in the tables) with animals of 30 days of age instead of the usual 20 days, utilizations of 38 ± 0.7% for 6 mice from 3 litters on the 20% dried skim milk diet at a calcium level of 0.28% and for 7 mice on the carrot diet an average of 32 ± 1.2% at an average level of 0.22% calcium intake were obtained. Because of the low calcium content of the dried carrots a very high percentage of carrots (over 50% of the diet) was necessary to replace 10 gm of dried skim milk calcium. This high proportion of carrots in the diet was not well tolerated by the mice. Therefore, in subsequent experiments (table 4), various levels of the vege-

tables were used. With the cabbage 10, 7 and 4 gm of the dried skim milk and with the carrots 7 gm instead of the usual 10 gm were replaced by the vegetable calcium.

The results (table 4) with the carrots bore out the general idea of the preliminary experiment that calcium of carrots is not as well utilized as milk calcium. Statistically this difference (43 as compared with 58%) proved definitely significant and it is felt that for mice, carrots are a poorer source of calcium than is milk. McCluggage and Mendel ('18) with

TABLE 4

The utilization of dietary calcium of weanling male albino mice[1] on diets containing 20% dried skim milk compared to the utilization on similar diets in which from 20 to 50% of the milk calcium is replaced by the calcium of dehydrated cabbage or carrots.

No.	DIETS		FOOD INTAKE	GAIN IN WEIGHT		CALCIUM	
	Dried skim milk	Dehydrated vegetables		Total	Per gm of food	Increment	Utilization factor
	%	%	*gm*	*gm*	*gm*	*mg*	%
M-2a	20		45.9	10.4	0.23	74	58 ± 1.0[2]
Cab-1	10	28[3]	53.3	10.0	0.19	66	45 ± 1.0
Cab-2	13	20[3]	56.5	11.3	0.20	75	48 ± 0.8
Cab-3	16	12[3]	55.1	12.1	0.22	77	50 ± 1.5
C-3	13	38[4]	49.1	8.6	0.17	58	54 ± 0.4

[1] All values are averages of 5 mice. All diets contained 0.28% calcium.

[2] Probable error of the mean.

[3] Cabbage.

[4] Carrots.

dogs, Shields, Fairbanks, Berryman and Mitchell ('40) with growing rats, and Breiter, Mills, Rutherford, Armstrong and Outhouse ('42) with adult humans, have also found this to be true. On the other hand, Rose ('20) and Edelstein ('32), working with adult humans and children, respectively, found carrots highly effective in maintaining calcium balances.

The generally lower utilization in the preliminary experiment of both milk (38 against 58%) and carrots (32 against 43%) is probably largely due to the differences in ages for as discussed later mice tend to show a poorer utilization of cal-

cium of cabbage as the age increases. The fact that the difference between the utilization of carrot and milk calcium in the preliminary experiment (32 against 38%) was much less than in the subsequent experiment of table 4 (43 against 58%) is due, at least in part, to the difference in the levels of dietary calcium, which as shown in table 3 significantly affects utilization values.

The dehydrated cabbage was fed at 3 levels of 12, 20 and 28% replacing 4, 7 and 10 gm of the 20 gm of skim milk, respectively. The utilization values of 50, 48, and 45% as against 58% for milk (table 4) show a definitely lower utilization of cabbage calcium. Also, as expected, a progressively lower utilization was observed as the per cent of milk replaced was increased. At the 13% dried skim milk level, the values of 43 $\pm$ 0.4% for carrots and the 48 $\pm$ 0.8% for cabbage show a slightly better utilization of cabbage calcium as compared to that of carrots. Kelly ('43) found that in the rat, Savoy cabbage calcium was 80% utilized as against 86% for milk, but fed both at relatively low levels of calcium intake.

A further attempt was made to investigate the apparent difference of utilization at various calcium levels noted previously (table 2). Four litters of mice were used as before except that 2 mice from each litter were killed as base line controls of initial calcium content. Four diets containing 10, 15, 20 and 25% dried skim milk were fed. The percentage utilization is inversely related to the calcium intake, as table 3 clearly shows.

A further attempt was made to check the previous observation that the age of the mice was an important factor in the percentage utilization of dietary calcium. As a few females were available, it was also decided to see if there were appreciable sex differences in utilization. Weanling mice from 2 litters (T and U) fed diet Cab-3 with a calcium content of 0.28% were used. The first group of males, T-2, T-3 and U-3, were killed after 12 days and showed utilization values of 61, 56 and 62%. The other males, T-4, and U-1, were killed after 24 days of feeding and showed values of 54 and 51% utiliza-

tion. Five males on this same diet (Cab-3) in a previous experiment (table 4) had shown an average value of 50 ± 1.5% over a 20-day period which is considered a good check between the 2 different experiments. The 2 females, T-1 and U-2, were killed after 24 days and showed slightly lower values of 46%.

TABLE 5

The calcium content of albino mice[1] under various conditions.

NO. OF MICE	SEX	AGE	TOTAL WEIGHT	BODY CALCIUM		REMARKS
		days	*gm*	*mg*	%	
5	..	0–1	5.3		0.292	newborn
2	..	15	10.3		0.577	sucklings
30	M	19–22			0.70 ± 0.008	weanlings
9	F	21–22			0.70 ± 0.017	weanlings
6	M	31			0.76 ± 0.009	1 week on 20% dried skim milk diet.
16	M	40			0.73 ± 0.007	3 weeks on 20% dried skim milk diet.
6	M	56			0.70 ± 0.005	5 weeks on 20% dried skim milk diet.
4(3F)		21			0.64 ± 0.007	weanlings of litter X
10(4F)		22			0.70 ± 0.006	weanlings of litter W
4	F	22			0.69 ± 0.007	weanling females of litter W
6	M	22			0.70 ± 0.007	weanling males of litter W
1	F	120	24.7	198.0	0.802	adult had just weaned 4 mice.
1	F	120	28.4	211.5	0.745	adult had weaned 6 mice 1 week before.
1	F	120	29.1	229.6	0.788	adult had weaned 7 mice 1 week before.
1	F	120	23.0	230.8	1.003	adult ate litter 3 weeks before.
1	F	120	23.9	239.7	1.002	adult ate litter 3 weeks before.
1	M	120	23.0	230.0	1.000	adult male.
1	M	120	24.7	249 6	1.010	adult male

[1] All animals after weaning had been on the basic 20% powdered skim milk diets (nos. M-2, M-2a).

Admittedly the number of animals used here is too small to show other than trends. However, the values are consistent and do bear out the observations in earlier experiments.

Table 5 includes data on the calcium content of albino mice at various ages. In general, the total content and percentage of calcium in the body increases from birth to adult life as Sherman and MacLeod ('25) and Briwa and Sherman ('41) found for rats. However, the data fail to show a significant sex difference for mice such as these authors found with rats.

SUMMARY AND CONCLUSIONS

The technique of the Sherman group for the determination of available calcium in vegetables using rats has been adapted to albino mice and, for our purposes, is considered to be superior as it results in a marked saving of space, time, materials and money in routine investigations of the availability of calcum of foodstuffs.

Litters of weanling albino mice at 20 days of age, in litter mate comparisons, were fed diets in which nearly all of the dietary calcium was derived from dried skim milk. The utilization values obtained were compared to those of similar diets in which up to one-half of the milk calcium was replaced by that of dehydrated vegetables, i.e., cabbage and carrots.

The mice utilized $45 \pm 1.0\%$ of the dietary calcium from the cabbage diet when one-half of the dried skim milk calcium was replaced by cabbage calcium as compared with a utilization of $58 \pm 1.0\%$ of the calcium from the control (20% dried skim milk) diet. When only 35% of the milk calcium was replaced by the vegetables tested, calcium utilizations of $48 \pm 0.8\%$ were obtained for the cabbage diet and $43 \pm 0.4\%$ for the carrot diet as compared with $58 \pm 1.0\%$ for the control diet.

The utilization of milk calcium was found to vary from $45 \pm 1.1\%$ at a 25% dried skim milk level (0.34% dietary calcium) to $86 \pm 3.7\%$ at a 10% skim milk level (0.17% dietary calcium).

There appeared to be a tendency for poorer utilization of dietary calcium as the age of the mice increased. A slightly poorer utilization by females was noted in the case of 2 females tested.

The data on total calcium content of albino mice showed values of about 0.3% at birth rising steadily to a little over 1% in adults, with no appreciable variation due to sex.

It was also apparent that weanling mice show a definitely lower utilization of dietary calcium than do weanling rats at a similar intake level.

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AMINO ACIDS IN THE URINE OF HUMAN SUBJECTS FED EGGS OR SOY BEANS[1]

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INTRODUCTION

The presence of amino acids in human urine has been recognized for many years but there has been relatively little information on the individual acids, largely because no suitable analytical methods were available for the quantitative determination of small amounts of these substances in complex mixtures. In the present study diets of known composition were fed to human subjects, and the urine collected was analyzed for 16 amino acids by established microbiological techniques (Schweigert et al., '44; Greenhut et al., '46; McMahan and Snell, '44).

EXPERIMENTAL

The diets were part of a large series designed to reveal differences in the biological availability of protein from soy beans at different stages of maturity and prepared in various ways. The general procedure was the same as that used by Murlin and coworkers ('38). Four human subjects (college women) consumed diets in which 80% of the total nitrogen was supplied by mature dried soy beans or by eggs, the

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"reference protein." The soy beans were prepared by soaking over night and boiling for 60 minutes, or by autoclaving at 110°C. for 30 minutes. The protein level of the diets supplied 5% of the total calories and the remaining calories were divided equally between carbohydrate and fat. Food furnishing approximately 45 cal. per kg of body weight was consumed daily. The daily intake of 3 of the 4 subjects is indicated in table 1. These subjects weighed an average of 64 kg; the fourth subject weighed only 56 kg and received 87% as much nitrogen as the others.

TABLE 1

Composition of diets consumed daily.

COMPONENT	DIET I	DIETS II AND III
	gm	*gm*
Egg	216	...
Mature dried soy beans	...	77 [1]
Butter	49	59
Sucrose	114	101
Cream	107	107
Cornstarch	75	75
Crisco	30	30
Karo	28	28
Lettuce	100	100
Carrots	50	50
Grapefruit juice	200	200
Applesauce	150	150
Lactose	70	70
Salad oil	10	10
Gm of nitrogen	5.17	5.13

[1] Diet II contained boiled soy beans; the soy beans in diet III were autoclaved.

The subjects consumed the diets for periods of 6 days each in the following sequence: egg protein diet I; boiled mature soy protein diet II; egg protein diet I; autoclaved mature soy protein diet III. Routine determinations of nitrogen balance were made throughout each of the experimental periods. The urine samples analyzed for amino acids were those collected on the sixth day of each of the last 3 dietary periods. The specimens from individual subjects were preserved under

toluene and HCl, and the total daily urine was diluted to 1500 ml, adjusted to a pH of 7, and then 15 ml were further diluted to 100 ml for the microbiological assays. Amino acids (tables 2–4) were then determined with *Lactobacillus arabinosus, Leuconostoc mesenteroides,* or *Streptococcus faecalis R* by methods previously employed on urine from experimental animals (Sauberlich and Baumann, '46).

RESULTS

The subjects were in good health on all diets. They maintained their weights and remained in nitrogen equilibrium for the second and third experimental periods although they went into negative nitrogen balance during the fourth period when autoclaved soy protein was fed (Steele, '46). An average of 5.13 gm of nitrogen was consumed daily during the latter period, while the average loss of nitrogen per day was 5.77 gm (5.31–6.26).

Fourteen of the 16 amino acids determined were found in most of the specimens of urine analyzed (tables 2 to 4). Leucine was always absent from the urine when the egg protein or the boiled soy bean protein was fed, but on the autoclaved soy bean diet, on which the subjects were in negative nitrogen balance, some leucine appeared in the urine of all subjects. Aspartic acid was never present in microbiologically available form in any of the specimens of urine. Occasional samples of urine were also devoid of isoleucine, lysine, or proline, although these latter amino acids were usually present and the average excretion of proline was one of the highest of all acids when expressed as the percentage of that ingested appearing in the urine. There were no significant variations in the excretion of amino acids by the different subjects nor did there appear to be any consistent relationship between the amounts of the different amino acids ingested and the amounts excreted in the "free" form in the urine. The most abundant amino acids in the egg diet were leucine, aspartic acid, and glutamic acid, and 2 of these did not appear in the urine as

TABLE 2

Urinary excretion of amino acids by 4 human subjects ingesting egg protein.

AMINO ACIDS INGESTED DAILY [1]		AMINO ACIDS EXCRETED DAILY		PERCENTAGE OF INGESTED AMINO ACIDS EXCRETED	
		Range	Average	Range	Average
	gm	*mg*	*mg*	%	%
Arginine	1.99	3.5– 5.0	4.4	0.17– 0.26	0.22
Aspartic acid (2)	3.38	0.0	0.0	0.0	0.0
Cystine	0.57	70.7–81.3	76.2	11.78–16.12	13.40
Glutamic acid (1)	3.32	30.0–93.0	61.0	0.86– 2.79	1.84
Histidine	1.12	51.4–84.0	74.6	4.44– 8.30	6.68
Isoleucine	1.59	0.0– 5.0	5.0	0.00– 0.30	0.31
Leucine	5.37	0.0–	0.0	0.00	0.00
Lysine	1.79	0.0– 6.0	1.5	0.00– 0.32	0.08
Methionine	0.98	0.8– 4.3	2.4	0.10– 0.52	0.25
Phenylalanine	1.69	3.2– 8.0	4.9	0.18– 0.45	0.29
Proline (1)	0.92	0.0–20.0	7.0	0.00– 2.08	0.51
Serine (2)	2.88	13.0–33.0	27.0	0.44– 1.16	0.95
Threonine	1.42	21.0–27.0	24.9	1.68– 1.82	1.75
Tryptophane	0.48	5.8– 8.2	7.5	1.16– 1.83	1.56
Tyrosine	1.51	13.0–25.0	16.5	0.82– 1.91	1.10
Valine	1.35	4.4–11.5	7.3	0.31– 0.82	0.55

[1] Average ingestion for the 4 subjects. The amounts of amino acids ingested daily were calculated from the percentages of amino acids in eggs as published in Block and Bolling ('45), except those marked (1) which are from Jacobs, M., Food and Food Products, vol. 1 ('44) and those marked (2) which were determined in this laboratory.

"free"[2] acids, while only 61 mg of the 3324 mg of glutamic acid ingested daily was excreted free in the urine. The most abundant amino acids in the urine were cystine and histidine, neither of which was particularly abundant in any of the diets fed.

[2] The term "free" amino acid is used to indicate those forms of the acid available to the species of microorganism used in the assay. This would include the amino acids in certain peptides, such as leucyl glycine which can satisfy the needs of the organism for these amino acids (Kuiken et al., '43), although the amino acids of many other peptides do not appear to be microbiologically available (Lewis and Olcott, '45).

Expressed as the percentage of ingested amino acid excreted in microbiologically available form, wide differences were observed between the different amino acids. Thus on the egg diet the percentage of ingested leucine and aspartic acid excreted was 0.0% and it was less than 0.5% for arginine, isoleucine, lysine, methionine, and phenylalanine. On the other hand, 13.4% of the ingested cystine appeared in the urine and 6.8% of the ingested histidine. These latter 2 amino acids were also the most abundant in the urines excreted on the soy bean diets (tables 3 and 4).

TABLE 3

Urinary excretion of amino acids by 4 human subjects ingesting protein of boiled soy beans.

AMINO ACIDS INGESTED DAILY [1]		AMINO ACIDS EXCRETED DAILY		PERCENTAGE OF INGESTED AMINO ACIDS EXCRETED	
		Range	Average	Range	Average
	gm	*mg*	*mg*	%	%
Arginine	1.67	4.5– 5.5	5.1	0.23– 0.37	0.36
Aspartic acid [3]	3.98	0.0	0.0	0.00–	0.00
Cystine	0.20	66.6–72.9	68.7	31.80–37.20	34.00
Glutamic acid [2]	4.32	25.0–44.0	33.2	0.56– 1.16	0.76
Histidine	1.06	55.9–87.5	73.2	5.14– 8.10	6.92
Isoleucine	1.42	0.0–10.0	4.5	0.00– 0.68	0.32
Leucine	2.05	0.0– 0.0	0.0	0.00	0.00
Lysine	1.62	0.0– 6.0	3.0	0.00– 0.41	0.37
Methionine	0.63	0.7– 4.5	2.5	0.12– 0.71	0.40
Phenylalanine	1.73	4.0–11.8	7.1	0.22– 0.66	0.41
Proline [1]	0.99	0.0–20.0	7.0	0.00– 2.30	0.60
Serine [2]	1.51	13.0–26.0	21.3	0.89– 1.66	1.41
Threonine	1.18	15.8–22.0	19.7	1.29– 1.80	1.67
Tryptophane	0.48	5.0– 9.0	7.5	1.16– 1.97	1.55
Tyrosine	1.26	8.0–14.0	11.0	0.61– 1.08	0.88
Valine	1.29	3.1– 5.0	4.1	0.23– 0.38	0.32

[1] Average ingestion for the 4 subjects. The amounts of amino acids ingested daily were calculated from the percentages of amino acids in eggs as published in Block and Bolling ('45), except those marked [1] which are from Jacobs, M., Food and Food Products, vol. 1 ('44), [3] Baumgarten et al. ('46), and those marked [3] which were determined in this laboratory.

When boiled soy beans were fed, the excretion of "free" amino acids was very similar qualitatively to that observed on the diet containing eggs, in spite of the different amino acid mixture that was present (tables 3 and 4). The soy bean diet contained more glutamic acid than the egg diet and presumably also more nitrogenous compounds other than the 16 amino acids determined, since the percentages of total nitrogen were essentially equal on both diets. The soy bean diet contained less leucine, less methionine, and appreciably less serine and cystine than the egg diet. Nevertheless the amounts of cystine excreted were essentially the same whether eggs or soy beans were fed. Since the daily intake of cystine was relatively low on the soy bean diets, the percentages of excretion appeared to be particularly high on the latter, 34.4 and 36.6% on boiled and autoclaved soy beans, respectively, as contrasted to 13.4% on eggs. With the single exception of leucine, there were no very marked differences between the excretion of amino acids during the periods representing the 2 kinds of soy beans (tables 3 and 4). No leucine appeared when the boiled soy bean diet was eaten, while 13.0 mg were excreted daily in the urine when autoclaved soy beans were fed. But if, as suggested above, the appearance of leucine on the latter diet was related to the negative nitrogen balance of the subjects, the mechanism for the excretion of leucine would still appear to differ from that for the other amino acids, since the excretion of the other acids was not altered greatly by the negative nitrogen balance (tables 3 and 4). Leucine has been reported to be present in certain pathological urines, presumably combined with tyrosine (Hawk and Bergeim, '44).

Significantly there were no essential differences in the excretion of methionine on the 3 diets, although methionine is apparently the limiting amino acid in raw soy beans, and the degree of availability depends on the heat treatment applied. Nor did the excretion of lysine appear to be in any respect unusual in these experiments. Lysine is relatively labile to heat (Greaves, Morgan and Loveen, '38) and a measurable

destruction of lysine has been reported in over-heated soy bean meal (Clandinin et al., '46). In the present experiments the daily average excretion of lysine was 1.5 mg during the autoclaved soy bean period and 3.0 mg on boiled soy beans. This

TABLE 4

Urinary excretion of amino acids by 4 human subjects ingesting protein of autoclaved soy beans.

AMINO ACIDS INGESTED DAILY [1]		AMINO ACIDS EXCRETED DAILY		PERCENTAGE OF INGESTED AMINO ACIDS EXCRETED	
		Range	Average	Range	Average
	gm	*mg*	*mg*	%	%
Arginine	1.67	6.0–10.0	8.1	0.35– 0.68	0.49
Aspartic acid [3]	3.98	0.0	0.0	0.0	0.0
Cystine	0.20	62.9–91.7	74.0	30.00–43.50	36.60
Glutamic acid [3]	4.32	23.0–70.0	49.0	0.51– 1.83	1.13
Histidine	1.06	61.8–77.0	69.3	5.70– 7.10	6.55
Isoleucine	1.42	2.0–12.0	4.5	0.14– 0.81	0.32
Leucine	2.05	6.0–23.0	13.0	0.28– 1.24	0.64
Lysine	1.62	0.0– 6.0	1.5	0.00– 0.41	0.10
Methionine	0.63	1.2–10.0	4.3	0.10– 1.54	0.68
Phenylalanine	1.73	2.0– 4.1	3.2	0.11– 0.27	0.19
Proline [1]	0.99	0.0–20.0	15.0	0.00– 2.30	2.02
Serine [2]	1.51	21.0–38.0	26.5	1.34– 2.78	1.75
Threonine	1.18	21.0–23.0	22.0	1.70– 2.18	1.87
Tryptophane	0.48	6.6–11.0	8.4	1.30– 2.20	1.74
Tyrosine	1.26	13.0–17.0	14.2	1.00– 1.30	1.09
Valine	1.29	6.0–11.5	9.2	0.45– 1.00	0.72

[1] Average ingestion for the 4 subjects. The amounts of amino acids ingested daily were calculated from the percentages of amino acids in eggs as published in Block and Bolling ('45), except those marked [1] which are from Jacobs, M., Food and Food Products, vol. 1 ('44), [2] Baumgarten et al. ('46), and those marked [3] which were determined in this laboratory.

difference might suggest a difference in lysine intake. On both diets, however, variations within groups were wide and in-individual samples of urine were encountered in which no lysine was present. Nevertheless, if the differences in individual excretion between the 2 groups should prove to be

significant, it would appear that boiling soy beans for 60 minutes was less deleterious to lysine than autoclaving at a higher temperature for a shorter period of time. It is even possible that the negative nitrogen balance of the subjects on the autoclaved beans may have been due to an insufficiency of available lysine. Murlin and co-workers ('46) added lysine to a baked soy bean diet and observed a higher biological value as tested on human subjects.

Peptides

Experiments on urine from rats and mice (Sauberlich and Baumann, '46) suggest that at least half of the amino acids in mouse urine is present as peptides, while in rat urine the percentage of such bound amino acids exceeds 75%. Preliminary experiments in the present study suggest that bound amino acids were also present in the samples of human urine analyzed. The urine was hydrolyzed in 2 N acid or 5 N $Ba(OH)_2$ for 5 hours at 15 pounds pressure and the samples were then re-assayed for certain of the amino acids. No leucine was found, although recoveries of leucine added prior to hydrolysis ranged from 97 to 133%. Hence it was concluded that this acid is not present in normal human urine in either the free form nor as a peptide. However, the amounts of microbiologically available tyrosine, tryptophane, and serine were found to have increased 4 to 5 fold after hydrolysis. A 24-hour specimen of urine which contained no aspartic acid before hydrolysis contained 88 mg of this acid after the peptides were hydrolyzed. Aspartic acid, therefore, is an example of an amino acid that exists in human urine almost entirely in a bound form. Several other workers have concluded that human urine contains peptides (Van Slyke, '13; and Albanese et al., '46).

DISCUSSION

In a previous study it was observed (a) that the amounts of the amino acids excreted in the urine by rats and mice de-

pended upon the amounts ingested, (b) that the percentages of ingested acid appearing in the urine in microbiologically available form were approximately the same for all amino acids except cystine and (c) that all of the 16 amino acids determined were present in every specimen of rat or mouse urine examined (Sauberlich and Baumann, '46).

The present results on human urine indicate that the excretion of amino acids by man differs from that by the rat or mouse in each of these respects. The amounts of any one amino acid excreted by human subjects were essentially the same whether eggs or boiled soy beans were fed, in spite of differences in the amino acid content of the two diets. Many specimens of human urine were encountered that lacked one or more of the amino acids proline, lysine, or isoleucine; free aspartic acid was never present, and leucine only under special circumstances. On the other hand histidine and cystine were always present in relatively high percentages of the amounts ingested.

These differences between species suggest that in general the human organism exerts more control than rats or mice over the excretion of amino acids into the urine, and furthermore that not all amino acids are controlled to the same extent. It is also possible that deamination reactions proceed more rapidly or completely in man than in the rat or mouse.

Folin and Berglund ('22) observed that the amounts of alpha amino nitrogen in the urine of men and dogs increased with increases in the blood, and concluded that there is no renal threshold for amino acids comparable to that for glucose. The colorimetric method of analysis employed (Folin, '22), however, lacks specificity, and does not measure all amino acids to the same extent. It is evident from the present data that "thresholds" for the amino acids do exist and that they vary from one amino acid to another. Presumably amino acids differ in the ease with which they can be reabsorbed from the glomerular filtrate through the renal tubules. Leucine is an example of an amino acid which must be very efficiently reabsorbed, since it does not appear in human urine, although

human blood contains from 17.3 to 25.7 μg per ml of plasma (Hier and Bergeim, '46). These amounts do not differ significantly from those for other essential amino acids in blood, including histidine, which occurs in urine in relatively large amounts.

The high urinary excretion of cystine observed requires some comment. In the previous study (Sauberlich and Baumann, '46) more cystine was found in the urine of rats and mice than the amounts of any other amino acid, but this result was not emphasized because the diet contained cystine in the free form while all other amino acids were present only as protein (casein). In the present experiments with human subjects, however, the cystine fed was all combined as protein, and again cystine was the most abundant amino acid in the urine. Cystine can function in the detoxication of many aromatic compounds (Stekol, '46) whence it appears in urine combined as mercapturic acids. Cystine was also one of the first free amino acids to be detected in urine (Alsberg and Folin, '05) and an abnormally high excretion of this amino acid has been noted more frequently than that of any other. It is possible, however, that microbiological methods of determination will reveal metabolic abnormalities similar to cystinuria but involving other acids. Polarographic determinations (Reed, '42) indicated an excretion of 40 to 80 mg of cystine — cysteine daily in urine from normal human males; in the present study daily microbiological values ranged from 60 to 91 mg. The latter method likewise measures both cystine and cysteine.

SUMMARY

1. Diets containing eggs or soy beans as sources of protein were fed to 4 human subjects at a protein level corresponding to 5% of the ingested calories, and the amino acid content of the urine was determined microbiologically.

2. Cystine was invariably found to be excreted in the highest percentage of the amount ingested, 12 to 44%. Histidine was next, 4 to 8%. The percentage excretion for most amino acids was found to range from about 0.2 to 1.8% of that ingested.

Proline, lysine, and isoleucine were frequently absent from the specimens of urine analyzed, free aspartic acid was always absent, while leucine was present only when the subjects were in negative balance (autoclaved soy bean diet).

3. There were no group differences between the excretion of essential and non-essential amino acids.

4. The hydrolysis of urine resulted in appreciable increases in microbiologically available tyrosine, serine, tryptophane, and aspartic acid. Apparently many amino acids in human urine were bound as peptides.

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THE BIOCHEMICAL DEFECT UNDERLYING THE NUTRITIONAL FAILURE OF YOUNG RATS ON DIETS CONTAINING EXCESSIVE QUANTITIES OF LACTOSE OR GALACTOSE

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The inadequacy of galactose as the carbohydrate of rat dietaries has been reported by Guha ('31), and of lactose by Ershoff and Deuel ('44), and Handler and Dubin ('46). Young rats, fed a diet in which lactose or galactose is the sole carbohydrate, fail to grow properly and die in 3–20 days. The present report describes several attempts which have been made to determine the physiological mechanism underlying this nutritional failure.

EXPERIMENTAL

All rats were males of the Vanderbilt strain (Wolfe, Bryan and Wright, '38). They were grown to a weight of 45 gm on a commercial stock chow after weaning, then housed in individual cages and offered the experimental diets. The basal diet (in %) consisted of casein 10, lactose (or other carbohydrate) 81, salts (Hubbell, Mendel and Wakeman, '37) 4, cod liver oil 2, cottonseed oil 3, corn oil 1. In addition each kilo of diet contained thiamine 3 mg, riboflavin 5 mg, pyridoxine 3 mg, calcium pantothenate 30 mg, nicotinic acid 5 mg, choline chloride 3 gm, inositol 2 gm, *p*-aminobenzoic acid 0.5 gm, mixed tocopherols 50 mg, and naphthohydroquinone acetate 50 mg.

Various modifications of this diet were fed to groups of 10 rats each. The results are summarized in table 1. It will be seen that galactose, or an equal mixture of glucose and galactose was as deleterious in its effects as lactose itself. This is in agreement with the work of Guha ('31) but in contrast to the findings of Ershoff and Deuel ('44). It would seem to indicate that the galactose moiety of the lactose molecule is responsible for the observed effects. Increasing the protein

TABLE 1

The effects of dietary modifications on lactose toxicity.

DIET	DIETARY MODIFICATION	WEIGHT CHANGE	SURVIVAL TIME
		gm	*days*
A	Sucrose 81%	23.0	14.0 [1]
B	Lactose 81% — diarrhea	— 19.1	4.9
	no diarrhea	— 12.7	7.3
C	Galactose 81% — diarrhea	— 18.3	5.2
	no diarrhea	— 15.6	6.8
D	Galactose 81% Glucose 40%	— 15.3	7.9
E	Lactose 60%	— 9.4	10.7
F	Lactose 40%	7.1	14.0 [1]
G	Galactose 20% Glucose 61%	6.8	... [2]
H	Casein 30% Lactose 61%	0.8	11.2
J	Lard 20% Lactose 61%	3.9	12.3
K	Sulfasuxidine 3% Lactose 78%	— 15.9	7.2
L	Cellulose 10% Lactose 71%	— 13.3	7.7
M	$NaHCO_3$ 0.5% Lactose 80.5%	— 16.1	6.8
N	Saline drinking water Lactose 81%	— 14.0	7.0

[1] Animals sacrificed after 2 weeks.

[2] Two rats died on eleventh day, remainder sacrificed after 2 weeks.

and/or fat content of the diet did increase the rats' survival time and at sufficiently high levels afforded complete protection. However, this does not seem necessarily to have been due to any specific ameliorating effect of the protein or fat but, rather, may perhaps be attributed to the diminished lactose ingestion. The critical level of lactose in the diet appeared to be about 60–70% while that of galactose was 30–40%. In agreement with Ershoff and Deuel ('44) and Guha ('31) it was found that when rats weighing more than

125 gm were offered the high lactose or galactose diets, they grew very slowly but survived for several months, developing the lenticular opacities typical of such diets, as indeed did many of the young rats before they died.

Since most rats showed at least a moderate diarrhea when fed the lactose diets (and to a lesser extent on the galactose diets as well) attempts were made to control this with both cellulose and sulfasuxidine. However, neither of these did effectively control the diarrhea nor did they appreciably lengthen the rats' survival time. Because of the possibility of dehydration and acidosis due to both the diarrhea and absorption of lactic acid from the bowel, one group was offered 0.4% NaCl in place of drinking water while 0.5% of $NaHCO_3$

TABLE 2

Blood constituents after lactose feeding.

DIETARY CARBOHYDRATE	SERUM PROTEINS	CO_2 CAPACITY	LACTIC ACID	PHOSPHATASE	CA	P	NPN
	gm %	*vol. %*	*mg %*	*B.U.*[1]	*mg %*		*mg %*
Sucrose	7.3	56	22	16.6	10.1	7.4	31
Lactose	6.9	52	25	31.0	12.4	6.3	44
Galactose	6.7	57	20	37.3	11.8	5.0	41

[1] Bodansky Units.

was added to the ration of another group. Again, neither of these measures increased the rats' survival time and actually, as will be seen later, no serious acidosis or dehydration did occur in lactose or galactose-fed animals.

Blood was obtained by decapitation of a number of moribund animals on the lactose and galactose diets and from controls on the sucrose diet. Analyses were performed for serum proteins, CO_2 combining power, lactic acid, phosphatase, calcium, phosphorus and non-protein nitrogen. The results are summarized in table 2. Each value represents the mean of a group of 12 rats although in most instances blood from a number of rats was pooled in order to obtain a quantity sufficient to perform the determination. From the hematocrit,

serum proteins and alkali reserve it was adjudged that no serious dehydration or acidosis resulted from either lactose or galactose feeding. While lactose may stimulate the development of an acidophilic intestinal flora the lactic acid was either lost in the stool or readily handled by the liver as its concentration did not rise in the systemic circulation nor did it provoke an acidosis. It is doubtful that the slightly elevated non-protein nitrogen values were of primary importance in the death of lactose or galactose-fed animals but represent a terminal event. The elevation of serum calcium is compatible with the known enhanced absorption of calcium from the intestine produced by lactose feeding while the decreased

TABLE 3

Urinary constituents during lactose feeding. Mean 24-hr. values per rat during third week of feeding. Initial rat weight 150 gm.

DIETARY CARBOHYDRATE	VOLUME	pH	TITRATABLE ACIDITY	NH_3	SUGAR	ACETONE BODIES	Ca	P
	ml		*ml 0.1 N NaOH*		*gm*		*mg*	*mg*
Sucrose	14	6.5	1.8	1.2	0	0	1.4	1.6
Lactose	43	5.9	5.7	6.4	0.48	0	9.2	1.9
Galactose	68	5.6	5.9	7.8	1.40	0	5.7	3.8

phosphorus may be associated either with the elevated serum calcium concentration or with the disturbed carbohydrate metabolism to be discussed later. A discussion of the increased serum alkaline phosphatase will be reserved for discussion in a later report (Handler, Follis and Baylin, in preparation).

Adolescent rats weighing 150 gm initially were placed in metabolism cages and urine was collected from pairs of rats. Three pairs of rats were placed on each of diets A, B and C. A 24-hour urine was collected from each pair every fourth day for 60 days. The urines were preserved with alcoholic thymol. The results are summarized in table 3. Each value is the mean of 18 samples collected from 3 pairs of rats.

The increased urine volume observed on the lactose and galactose diets was the result of the quite considerable excretion of reducing sugar seen in these animals. The values expressed in the table were calculated as galactose, and indeed, since acid hydrolysis failed to increase the reducing power of the urine from lactose-fed rats it would seem that these animals excreted no lactose. Osazones prepared from the urines of lactose and galactose-fed rats appeared identical in crystal structure with an authentic sample of galactosazone and gave no depression of the melting point in mixed melting point determinations. However, in no case was the sugar excretion of sufficient magnitude to impose a serious caloric want. This was also true in young animals as well.

A tendency toward acidosis was certainly indicated by the acid urines and high excretion of titratable acid and ammonia by rats on both the lactose and galactose diets. While this might be thought to be due at least in part to the diarrhea, these acid urines were also encountered from rats producing reasonably well-formed stools and so it seems not unlikely that the tendency to acidosis was occasioned by the production and excretion of some unidentified organic acid other than lactic, pyruvic, acetoacetic or β-hydroxybutyric. Since no diminution in the alkaline reserve was found, the renal excretion of this acid adequately prevented a systemic acidosis.

The markedly increased excretion of calcium by both lactose and galactose-fed rats is in keeping with the known enhanced intestinal absorption of calcium produced by lactose ingestion. Since complete balance studies were not performed it is not possible to state whether there occurred any unusually great retention of calcium or even, perhaps, actual bone demineralization.

Complete autopsies were performed on 4 rats fed galactose diet C and 4 fed lactose diet B. The animals were sacrificed when moribund, the tissues fixed in 10% neutral formalin, and sections were stained with hematoxylin and eosin. Heart, lungs, liver, spleen, pancreas, kidney, adrenal, stomach, intestine, brain, thyroid and bone were examined. However,

nothing was found which seemed contributory to an understanding of the disease process. The intestines were greatly distended with gas and contained heavy bacterial colonies. No intussusception, volvulus, or ulceration was noted. The lymphoid tissues, particularly spleen, all appeared atrophic as they do in general inanition. Blood samples removed from such animals under sterile conditions failed to reveal any bacteremia. Despite the unusual turnover of calcium and the somewhat elevated serum calcium concentrations, no evidence of calcification of soft tissues, blood vessels or kidney was found. The latter is also in keeping with the essentially normal blood NPN values. X-ray examination revealed only a complete failure of skeletal calcification in adolescent rats maintained on lactose or galactose. This will be reported and discussed in detail in a subsequent publication (Handler, Follis and Baylin).

Bonnamour and Escallon ('13) found a diminished calcium and increased phosphorus content in the femur of a rabbit which had been given 10 gm of lactose intravenously daily for 3 months. While it seemed unlikely that this sort of process, if real, could have accounted for the large urinary calcium excretions found in the present animals, it was thought of interest to study the effects of intraperitoneally administered glucose, sucrose, lactose, galactose and β-lactose. Each of these was given as 10 ml of a 5% solution daily to groups of 3 rats whose initial weight was 150 gm each and which were maintained on the basal sucrose-containing diet A. Twice each week for 2 months, 24-hour urines were collected and calcium, phosphorus and sugar excretion determined. No differences were noted in the various groups and, other than the urine volumes and sugar excretion, they did not differ essentially from a control group given 10 ml of isotonic saline per day. At the end of the experimental period the animals were sacrificed, their femurs removed, soaked in 95% ethyl alcohol for 24 hours and then in ether for 12 hours. They were then dried in an oven, weighed, digested with hot 0.1 N HCl for 2 hours, made to volume after which aliquots were taken for

calcium and phosphorus analyses. While the femurs of the lactose, β-lactose and galactose injected rats weighed slightly less than those given glucose, sucrose or saline, the calcium and phosphorus concentrations and the Ca/P ratios were not significantly different. These results are not in agreement with the report of Bonnamour and Escallon ('13) on a rabbit, and under these conditions, intraperitoneal administration of these various carbohydrates for 2 months did not produce any significant decalcification of rat femurs.

TABLE 4

Carbohydrate metabolism on lactose and galactose diets.
(Each value mean of 6 rats)

INITIAL RAT WEIGHT	TIME	DIETARY CARBOHYDRATE	BLOOD GLUCOSE	BLOOD GALACTOSE	SERUM P	LIVER GLYCOGEN
gm	*days*		*mg %*	*mg %*	*mg %*	*%*
50	4	Sucrose	112	...	6.5	3.4
50	4	Lactose	89	106	5.4	1.7
50	4	Galactose	78	285	4.8	2.5
50	7	Sucrose	122	...	7.4	3.5
50	7	Lactose	67	320	6.0	0.7
50	7	Galactose	48	473	4.6	0.4
150	10	Sucrose	97	...	8.3	3.0
150	10	Lactose	83	98	6.9	1.3
150	10	Galactose	87	316	5.1	3.1

Carbohydrate metabolism studies were performed in the following manner. Blood and liver samples were obtained from sacrificed young rats after 4 days on diets B (lactose) and C (galactose) and from other rats on these diets when they appeared moribund (6–9 days). Control animals receiving diet A were sacrificed after 4 and 7 days. Samples were also obtained from 150-gm rats after they had been on diets A, B, and C for 10 days. Total reducing sugar, "true glucose" (Somogyi, '27) and phosphorus analyses were performed on the blood samples, and glycogen was determined in the liver (Good, Kramer and Somoygi, '33). The results are summarized in table 4.

Both lactose and galactose feeding consistently resulted in a lowering of the blood true glucose, the serum phosphorus and liver glycogen in young rats. The effect of galactose on the serum phosphorus was much more marked than was that of lactose. Liver glycogen values were actually lower in the lactose than in galactose-fed adolescent rats and the young ones sacrificed after 4 days but not in moribund young rats. This may perhaps have been due to the poorer state of these animals occasioned by the severe diarrhea. Probably the most significant finding was the reduced true glucose values obtained in the moribund rats on both lactose and galactose diets.

DISCUSSION

From the data presented herein and the studies of Ershoff and Deuel ('44) and Guha ('31) there is little doubt but that high concentrations of lactose in the diet are toxic to the rat. However, the physiological mechanism by which this toxicity is exerted has not yet been completely resolved. From the present work and that of Guha it would appear that it is the galactose moiety of the lactose molecule which is responsible for the observed effects. This, however, is contraindicated by the fact that while both lactose and β-lactose proved toxic in the diets of rats of the Long-Evans and the U.S.C. strains, equal amounts of galactose resulted only in a temporary weight loss followed by slow growth (Ershoff and Deuel, '44). Nevertheless, all indications in the present work seem to implicate galactose per se as the toxic factor.

Ershoff and Deuel ('44) concluded that the fatal effects of dietary lactose were probably dependent upon events in the intestine. It does not seem reasonable that, by changing the intestinal flora, lactose removed or diminished the supply of some, as yet unknown, dietary factor so essential to the animal economy that death resulted in as little as 3 days. Apparently not considered by previous workers was the possibility of death due to diarrhea with consequent acidosis and dehydration. However, this too has been ruled out since

blood studies showed no evidence of acidosis or dehydration nor did saline drinking water or $NaHCO_3$ prove therapeutically effective. Moreover, many rats, particularly those on the galactose diets, died in a few days yet showed reasonably well-formed stools during this period.

The effects of dietary lactose on calcium metabolism also seemed a possible etiologic factor in the death of these animals. Lactose, by stimulating the development of an acidophilic intestinal flora (Rettger and Cheplin, '23; Hudson and Parr, '24) lowers the pH of the intestinal tract below the duodenum (Robinson and Duncan, '31). It may be this phenomenon which is responsible for the enhanced intestinal absorption of calcium (Robinson and Duncan, '31). It seemed possible that there might exist a rational correlation between our observation that young rats die while adults survive indefinitely on lactose diets and the finding of French and Cowgill ('37), that in both rats and dogs lactose promotes calcium absorption in young animals but not in adults. While the markedly increased urinary excretion of calcium and slightly elevated serum calcium concentrations were compatible with the aforementioned effects of lactose, there were no indications that these changes were of any significance in the pathogenesis of this disease. Further, death appeared to be due to the presence of galactose, and the effects of galactose on calcium metabolism observed in the present work were not as marked as those of lactose.

Within 24 hours after being offered a lactose-containing diet virtually all rats showed the gross symptomatology described by Ershoff and Deuel ('44). They exhibited ruffled fur, edema of the hind paws, occasionally alopecia and were all generally filthy in appearance. This would all appear to be referable to the diarrhea and was only seen in those rats receiving galactose which also were afflicted with diarrhea. However, no animal actually appeared moribund, weak and listless on either lactose or galactose diets until there occurred a fall in the blood glucose concentration (table 4).

Guha ('31) concluded that the fatal results of galactose feeding were due to starvation. This was based upon the known slow utilization of galactose, its relatively low renal threshold and the increased appetite of rats on a high galactose diet. It is not possible to completely deny this possibility, but it does not seem likely when one considers that galactose-fed rats, eating as much as 7 gm a day excreted no more than 2.2 gm of galactose in the urine. If the remaining dietary galactose had been utilized properly such rats should survive almost indefinitely although their growth rate would be expected to be below normal. Instead they died in approximately the same time as did rats permitted access only to water. The possibility that death is due to simple starvation is also compatible with the observations of Holt and Kajdi ('44) that starving rats offered only lactose or galactose died as rapidly as did the unsupplemented controls while glucose, fructose, sucrose and various reasonably purified fats prolonged the rats' survival time as much as 25 days. Nevertheless, the extremely rapid course of the disease, the lack of any specific histological findings, the numbers of animals which died even more rapidly than do rats suffering from simple inanition, and the rather unexpected findings of Holt and Kajdi ('44) with respect to lactose and galactose described above, all point to the probability that death was due to some fundamental metabolic disturbance, presumably of carbohydrate metabolism. The findings in the present work suggest that not only was there a failure to utilize fully and properly the dietary lactose and galactose but that, perhaps, these actually interfered with normal carbohydrate (glycogen or glucose) metabolism. The studies reported herein do not permit any definitive statement of the nature of this disturbance but do indicate that galactose in the amounts given here may interfere with normal glucose metabolism. Thus, despite the presence of blood galactose concentrations as high as 600 mg %, moribund rats on lactose and galactose diets were found to have blood glucose concentrations as low as 40 mg % while the supply of liver glycogen appeared to be virtually ex-

hausted. It may be that this is related to the observation that liver glycogen after galactose feeding is built of 18 hexose units rather than the 12 units found in liver glycogen after glucose feeding (Bell, '36).

This situation appears to be analogous to that in infants showing galactosemia and cataracts (Bruck and Rapoport, '45; Goldbloom and Brickman, '46). In these children also were found high blood galactose levels and relatively low blood glucose concentrations as long as they were maintained on milk. When additional galactose was administered in tolerance tests, the blood galactose concentrations were elevated still further while the blood glucose fell to levels as low as 40 mg %. It should be noted that the blood glucose of normal children also fell to as low as 50 mg % during galactose tolerance tests. However, the blood glucose returned to normal and the blood galactose disappeared considerably more rapidly in the normal children than in the infants with "idiopathic galactosemia." Further, the clinical signs and symptoms of this state all appeared to be referable to the disturbance in carbohydrate metabolism and disappeared when the patients were placed on a lactose-free regime.

From consideration of the behavior of the rats described in the present report and the response of normal children to galactose tolerance tests it appears that the galactosemia with its untoward sequelae observed in some children as long as their diets contain lactose (as milk) is not really a qualitative idiopathic disturbance but rather a somewhat exaggerated quantitative response similar to that which can be obtained in normal rats and children if the galactose intake is sufficiently great.

SUMMARY

Weanling rats of the Vanderbilt strain die within 3 to 17 days after being placed on diets containing more than 60% lactose or 40% galactose. No lesions other than those associated with simple inanition were found by histological examination of their tissues. While most rats exhibited a

marked diarrhea and all a profound diuresis, death did not appear to have been due to dehydration or acidosis. While all rats had a marked calcinuria due to the enhanced calcium absorption from the intestine, their serum calcium concentrations were only moderately elevated and did not appear to be an etiologic factor in the death of such rats. Moribund rats on both lactose and galactose diets were found to have blood galactose concentrations varying from 210 to 640 mg % with true blood glucose levels which varied from 26 to 73 mg %. Simultaneously there occurred an appreciable drop in the serum inorganic phosphorus concentration and almost complete depletion of the liver glycogen. It is concluded that this disturbance of carbohydrate metabolism, which is similar to that occasionally observed in infants while they drink milk, is the prime etiologic factor in the death of rats on such diets. The occasional "galactosemia" seen in infants is thought to represent not a qualitative idiopathic phenomenon but a quantitative exaggeration of events which can be elicited in a normal animal when the galactose intake is sufficiently high. The mechanism whereby galactose interferes with normal glucose metabolism has not yet been clearly defined.

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EFFECT OF COOKING AND CURING ON LYSINE CONTENT OF PORK LUNCHEON MEAT

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THREE FIGURES

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The well-known reaction between nitrous acid and primary amines has led to the question of whether the "nitrite cure" impairs the lysine value of pork. It has been suggested that lysine would be among the first of the amino acids to be attacked if there were any reactions between individual amino acids in the proteins of meat, and the nitrite used in the curing process. In organic chemistry, reactions between primary amines and nitrous acid are ordinarily carried out in strongly acid solutions. The pH of meat never reaches such high acid values, but the high temperature reached during the heat treatment — about 112° to 113°C. — might favor the reaction.

Heat alone, particularly dry heat or toasting, has been shown to have an injurious effect on many proteins. Invaldsen ('29), Daniel and McCollum ('31), Morgan ('31), Maynard and Tunison ('32), Schneider ('32), Maynard, Bender and McCay ('32), Fixen and Jackson ('32), Fairbanks and Mitchell ('35), Murlin, Nasset and Walsh ('38), Stewart, Hensley and Peters ('43), Block, Cannon et al. ('46) and others have demonstrated that dry heat or toasting has an injurious effect on many proteins; and Greaves, Morgan and Loveen ('38) and Block, Cannon et al. ('46) have shown that the lysine in the protein molecule is particularly sensitive to

relatively mild processing procedures. In the cooking of meat, the temperature would probably not go as high as in a toasting process, but Morgan and Kern ('34) have reported that cooking by 3 different processes tended to lower the biological value of beef.

The commercial canning of luncheon meat involves both a nitrite cure and cooking, both of which might have some effect on the lysine content of the protein. If they have any effect on lysine, it may not be a destruction since Block, Jones and Gersdorff ('34) have reported that as much lysine could be isolated after acid hydrolysis of heated casein as from unheated, and Seegers and Mattill ('35) found that acid hydrolysis of heat impaired proteins restored their nutritive value when tryptophane, alone, was added.

The paucity of knowledge at the present time regarding the interactions of lysine in the protein molecule makes it difficult to interpret results secured by any one method of analysis. Animal feeding trials are still the ultimate tests for the availability of any one essential amino acid. Accordingly, animal feeding trials were conducted to get information on the relative nutritive values of fresh and processed pork luncheon meat, and to determine if there is any destruction of lysine during the cooking or curing process.

EXPERIMENTAL PROCEDURE

A representative batch of ground pork was obtained from a batch prepared for commercial production of canned pork luncheon meat. It was divided into 3 parts: one part was frozen as fresh meat, a second part was canned, and the third part was cured by a standard commercial method and then canned. Both of the canned lots were heat processed at the temperature and pressure used commercially in the packing plant. The curing ingredients included 0.15 pounds of sodium nitrite per 1000 pounds of meat. After curing and remixing, the meat was stuffed into 12-ounce cans and closed under vacuum in a commercial closing machine. The cooked samples

were heated in a retort for 1 hour at a temperature of 112° to 113°C., and then immediately cooled with cold water.

The meats were all stored in a refrigerator, the fresh meats being kept frozen until they were fed to the animals. When needed, a can was removed and opened, the meat ground twice through a small meat grinder, and mixed.

The 3 lots of meat were fed to young weanling rats as supplements to a basal ration composed of (in %) ground yellow corn 85.6, hydrogenated vegetable oil 9.5, salt mixture 4, sardine oil 0.5, l(−) cystine 0.2, and l(−) tryptophane 0.2 parts, respectively. In addition, each rat received 0.2 ml daily of a commercial preparation [1] as a source of the B vitamins. Previous (unpublished) feeding trials have shown that the greatest deficiency in this ration is with regard to the lysine content, and that young rats consuming the ration will grow but only very slowly. However, when the ration is supplemented with 0.2% l(+) lysine, growth is very markedly increased. It was calculated that when the rats were allowed 10 gm of the basal ration daily, 50% or more of the animals' requirements for amino acids were met, with the exception of the lysine requirement, where only about 20% of the requirement was supplied. Lysine, then, was the first limiting factor.

Ten rats were maintained on this diet throughout the experiment as negative controls. The other rats were divided into groups of 10 animals each, and given a daily supplement of 0.5 or 1.0 gm of the 3 meats, respectively, fresh, cooked, or cured-cooked. The basal ration was limited to 10 gm daily, and fed in the regular feed cup. The vitamin B supplement was pipetted into a small dish daily, and the meats were weighed out daily for each individual rat and dropped into the feed cup. Daily feed consumption records and weekly rat weight records were kept for a period of 8 weeks.

One can of meat from each of the three groups was dried, defatted, and analyzed for lysine by the specific decarboxylase method of Hanke ('46).

[1] Vitab.

DISCUSSION OF RESULTS

Growth curves showing gain in weight of the rats are presented in figures 1, 2 and 3. The rats on the basal ration alone gained very slowly, averaging only 22 gm in 8 weeks. When this same ration was supplemented with 0.2% of l(+) lysine, growth was increased to 82.5 gm in 8 weeks (0.2% l(+) lysine in the basal ration is equivalent to 20 mg of lysine when the rats are allowed 10 gm of food daily). The depression of the rate of gain in weight observed by Mitchell and Smutts ('32) when tryptophane is added to a corn ration does not appear to be a factor when lysine is also added.

When the meats were fed at a level of 0.5 gm daily as a supplement to the basal ration, growth was substantially increased over that observed in animals receiving no supplementary meat or lysine (figs. 1 and 2), and a further increase was observed in the rats fed 1.0 gm of the meats daily (fig. 3).

The addition of the meats to the rat diets added considerable amounts of other animo acids that were in short supply in the basal diet, but the increases in growth were in no case as high as was observed when the basal ration was supplemented with 0.2% of l(+) lysine. The amount of lysine added to the rats' daily diet through the meats (table 1) was in no case as high as the 20 mg consumed daily by the control group, hence, the limiting factor for growth was still lysine when these low levels of meat were fed.

Figure 1 presents the gain in weight obtained by feeding 0.5 gm of the meats daily. While the growth observed on the cured-cooked meat is slightly less than on the other meats, it is not regarded as significant since in another series of rats fed the same rations (fig. 2) growth on the cured-cooked meat was as high as that observed on the cooked, and higher than that observed on the fresh meat. The growth observed on rats fed the cooked meats, in general, runs slightly higher than any of the others, even when 1.0 gm daily was fed. This may be due in part at least, to the protein content of the meat. In the cooking process, a certain amount of water and fat separated from the uncured meat, and proved to be very difficult

to remix into the meat when the cans were opened. Some of the water was sometimes lost, which could account for the variation in the protein percentage from can to can. The fresh meat ranged from 15.6 to 16.2% protein, the cooked meat from 16.6 to 18.8, and the cured-cooked meat from 15.3 to 15.8% protein.

Figure 3, showing the gain in weight of rats receiving 1.0 gm of meat daily, indicates that the lysine even in this amount of meat is inadequate to support maximum growth, hence, if

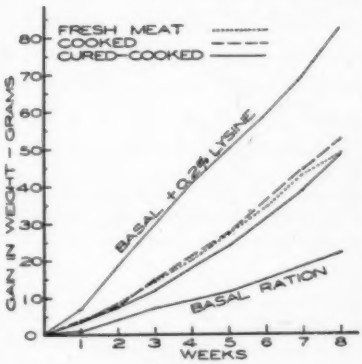

Figure 1

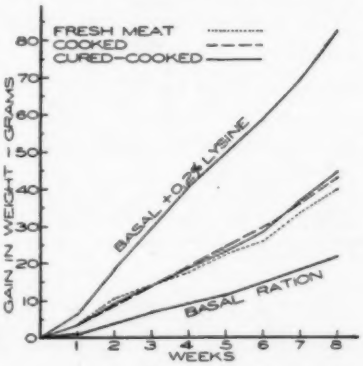

Figure 2

Fig. 1 Curves showing growth of rats receiving the lysine deficient basal ration, and daily supplements of 0.5 gm fresh (frozen), cooked, or cured-cooked pork luncheon meat, and 0.2% l(+) lysine (first series).

Fig. 2 Curves showing growth of a second series of rats fed the same supplements as in figure 1.

there were any significant differences in the lysine content of the 3 meats, it should appear in the growth curves of the rats receiving that amount or less of meat.

The lysine analysis of the 3 meats (table 1) indicates that there was some destruction of lysine in the cured-cooked meat but not in the cooked. When calculated to a 16% nitrogen basis, the difference between the lysine in the fresh (7.97%) and the cooked (8.06%) is not significant. The difference between the cured-cooked (7.01%) and the other two probably is significant, the loss of lysine being about 12%.

When the amounts of lysine are calculated, that each animal received per day in its meat supplement, the results can be correlated with the gains in weight shown in figure 1, but

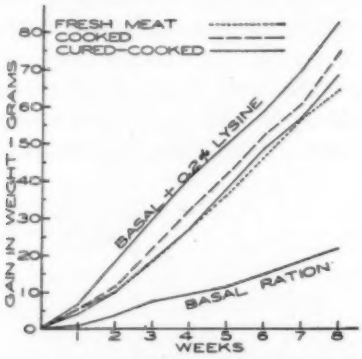

Fig. 3 Curves showing growth of rats receiving the lysine deficient basal ration, and daily supplements of 1.0 gm of fresh (frozen), cooked, or cured-cooked pork luncheon meat in comparison with 0.2% l(+) lysine.

TABLE 1

Lysine analysis of meats.

	FRESH MEAT	COOKED MEAT	CURED-COOKED MEAT
Lysine in meat calculated to 16% nitrogen basis — %	7.97	8.06	7.01
Average protein content of meat (N × 6.25) — %	15.9	17.7	15.5
Average lysine in meat as fed — %	1.267	1.426	1.086
Lysine consumed per day, 0.5 gm. Meat level — mg	6.33	7.13	5.43
Lysine consumed per day, 1.0 gm. Meat level — mg	12.67	14.26	10.86

not so easily with the weight gains of other animals shown in figures 2 and 3.

Since the gains in weight observed in the rats on the 3 samples of meat are so nearly uniform at any one level of

meat fed, it seems highly improbable that there could have been any destruction of lysine greater than the 12% shown by the analyses. Insofar as the animal is concerned, the destruction may have been either insignificant at that level, or the destruction may have been less in other cans of the same meats that were opened and fed to the animals.

SUMMARY AND CONCLUSIONS

Pork luncheon meats were analyzed for lysine, and fed to rats as a supplement to a diet which was deficient in lysine.

Lysine analyses of fresh, cooked, and cured-cooked pork luncheon meat indicate that there was no destruction of lysine due to the cooking, but the cured-cooked sample showed a loss of 12% of the original lysine content.

No significant differences in growth were observed on rats fed the 3 samples of meat, indicating that if there was a destruction of lysine, it was too small to detect in the feeding tests with rats.

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THE INFLUENCE OF PTEROYLGLUTAMIC ACID (A MEMBER OF THE VITAMIN M GROUP) ON THE ABSORPTION OF VITAMIN A AND CAROTENE BY PATIENTS WITH SPRUE[1]

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ONE FIGURE

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In diseases such as sprue, celiac disease, and cystic fibrosis of the pancreas, in which there is an impairment of the absorption of fat from the gastrointestinal tract, serum levels of carotene and vitamin A may be decreased. Also, the rise in serum vitamin A following the oral administration of a test dose of vitamin A may be absent or delayed (Chesney and McCoord, '33–'34; Clausen and McCoord, '38; Breese and McCoord, '39; May and McCreary, '41; May, McCreary and Blackfan, '42; Ingelfinger, '43; Adlersberg and Sobotka, '43; Cayer, Ruffin and Perlzweig, '45). Other findings which suggest a defective absorption of fat soluble factors in sprue are a hypoprothrombinemia (Fanconi, '38; Butt and Snell, '41; and Ingelfinger, '43) and a lowering of serum calcium, which may be due in part to a failure to absorb vitamin D (Bennett, Hunter and Vaughan, '32; Hanes, '43; Ingelfinger, '43). More recently it has been found that plasma concentrations of the

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other fat-soluble vitamin, tocopherol, is lowered in steatorrheas (Darby, Cherrington and Ruffin, '46).

The many similarities of sprue in man and of vitamin M deficiency in the monkey led to the now well-confirmed demonstration that pteroylglutamic acid (PGA) is active in producing hematologic and clinical remissions in sprue (Darby and Jones, '45; Moore, Bierbaum, Welch and Wright, '45; Darby, Jones and Johnson, '46a, b; Spies, Lopez, Menendez, Minnich and Koch, '46; Spies, Milanes, Menendez, Koch and Minnich, '46). We have reported that oral glucose tolerance curves and vitamin A absorption curves became more nearly normal in sprue after treatment with PGA (Darby and Jones, '45; Darby, Jones and Johnson, '46a, b). At the time of these earlier reports sufficient time had not elapsed to evaluate with finality the effectiveness of this vitamin on the absorption of fat and fat-soluble factors. The data now indicate that treatment with PGA results in a definite decrease in the total fat loss in the feces of patients with sprue (Darby and Jones, unpublished observations). It is of importance, therefore, to determine whether there also occurs evidence of improved absorption of fat-soluble vitamin A in the cases of sprue treated with PGA.

EXPERIMENTAL

The subjects were 4 patients with sprue observed in the Vanderbilt University Hospital. Certain data on 3 of the cases were previously reported (Darby, Jones and Johnson, '46a, b). After diagnostic studies were completed the patients were treated with doses of either 5 or 15 mg daily of pteroylglutamic acid [2] administered either orally or intramuscularly. In 2 cases (J.D. and P.S.) therapy was discontinued after 14 and 19 days, in the others it has been continuous. One of the former patients has relapsed following the withdrawal of therapy, and a second remission has now been

[2] We are indebted to Dr. Stanton M. Hardy, Medical Director, Lederle Laboratories, Inc., for generous supplies of synthetic pteroylglutamic acid used in these studies.

induced by treatment with PGA. None of the patients have received supplements of carotene or vitamin A, and there has been no effort to alter their usual diets.

TABLE 1

The effect of pteroylglutamic acid (PGA) on serum carotene and vitamin A levels in sprue.

PATIENT	DATE	CAROTENE	VITAMIN A	REMARKS
		μg/100 ml	*I.U./100 ml*	
J.D.	9-25-45	8	98	Before therapy
	9-29-45	18	95	Before therapy
	10- 4-45	..	..	Therapy instituted, 15 mg PGA daily, parenterally
	10-19-45	21	129	Remission
	10-22-45	..		Therapy discontinued
	11-21-45	22	133	Remission
	1-31-46	52	153	Remission
	3-20-46	72	158	Remission
	6-19-46	10	90	In relapse
	7-13-46	14	..	In relapse
	7-23-46	17	66	
	7-25-46	..	..	Therapy instituted, 5 mg PGA daily by mouth
	8-26-46	54	170	In remission
P.S.	10-18-45	12	90	Before therapy
	10-19-45	..	..	Therapy instituted, 15 mg PGA daily, parenterally
	10-22-45	19	60	
	11- 8-45	..	..	Therapy discontinued
	1- 3-46	63	66	Remission
P.B.	11- 9-45	21	119	Chronic sprue, before therapy
	11-10-45	..	..	Therapy instituted, 15 mg PGA daily, parenterally
	11-21-45	25	101	Remission
	12- 6-45	13	89	Remission
	7-17-46	65	124	Remission
W.O.	5- 6-46	..	..	Therapy instituted, 5 mg daily, orally
	5-16-46	36	133	Early remission
	5-25-46	73	103	Remission
	7-13-46	51	95	Remission

Serum carotene was estimated by measuring the absorption of a petroleum ether extract in an Evelyn photoelectric colorimeter at 440 μ and vitamin A was determined by means of the Carr-Price reaction as described by Kaser and Stekol ('43). Vitamin A tolerance tests were carried out by estimating the serum levels at intervals after the oral administration of 200,000 I.U. of vitamin A as a fish liver oil concentrate.

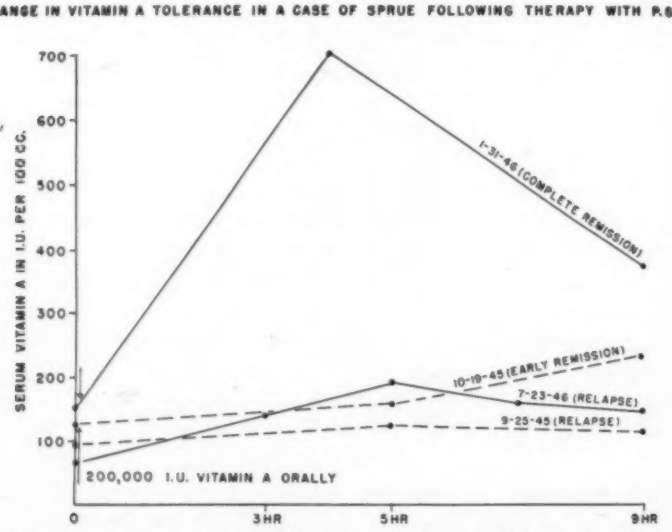

Fig. 1 Serum vitamin A levels following an oral dose of 200,000 I.U. of vitamin A to a patient with sprue. The slight increases observed on 3 occasions during relapse or early remission are in sharp contrast to the high rise observed during a complete remission following therapy with PGA.

RESULTS

The values for serum carotene and vitamin A are listed in table 1. Presumably the presence of adequate liver stores of vitamin A before the onset of sprue accounts for the satisfactory levels of vitamin A as found in these patients. All 4 subjects showed initial pronounced decreases in the serum carotene levels and a gradual increase in the carotene concen-

tration followed therapy with PGA. In one case (J.D.) these changes recurred during a second relapse and remission. It should be noted that as J.D. relapsed the carotene, which had approached a satisfactory level, again almost disappeared from his blood. There was also a simultaneous drop in tocopherol levels of the serum in this patient (Darby, Cherrington and Ruffin, '46).

Figure 1 depicts the vitamin A absorption curves for J.D. before treatment, during his clinical remission, and during the subsequent relapse. The profound change in the vitamin A absorption curves following therapy with PGA is apparent.

DISCUSSION

These results, together with those previously reported, indicate that a deficiency in man of the vitamin-M group, of which PGA is a member, results in defects of gastrointestinal absorption which may be reversed by supplying the deficient vitamin. The early cessation of the diarrhea, the reduction of the fat content of the stools, the return of the flat glucose tolerance curve to a normal one, and the rapid gain in weight in the patient with sprue treated with PGA are all evidences of improved absorption. In view of these changes the more slowly occurring increases in serum carotene concentration and the return of the vitamin A tolerance curve to normal during remission are consistent with the interpretation that PGA also favorably influences the absorption of carotene and vitamin A in sprue. The slower rate of correction of the absorption of fat-soluble substances than of water-soluble substances following the treatment of sprue with PGA is consistent with the more gradual disappearance of the steatorrhea in sprue after therapy with liver (Hanes, '43) and with *L. casei* factor (Darby, Jones and Johnson, '46b).

This evidence that the administration of one synthetic vitamin (PGA) alone may so greatly influence the concentration of another vitamin within the body may be cited as another example of an interrelationship of foodstuffs (Mitchell, '43; Moore, '45). It is impressive that a deficiency of vitamin M, as

seen in clinical sprue, results in demonstrable malabsorption of so many dietary factors — carbohydrate, fat, vitamins, and calcium. Additional experimental studies would seem to be indicated in order to determine whether any of the secondary deficiencies observed in the sulfa-treated ("vitamin M deficient") rat (Daft and Sebrell, '45) are due to impaired absorption. The gastrointestinal defects reported by McCarrison ('21) in his "vitamin B deficient" monkeys have been interpreted as due to a deficiency of thiamine (Williams and Spies, '38). It is now held that McCarrison's monkeys presented the clinical picture of vitamin M deficiency (Day, '44). It appears, therefore, that a reinvestigation of the relative roles of thiamine and of the members of the vitamin M group in maintaining gastrointestinal function would be timely.

SUMMARY

Serum carotene and vitamin A levels in 4 cases of sprue treated with pteroylglutamic acid (PGA) are reported. It was found that the low serum carotene levels observed in relapse were gradually increased following therapy. The carotene returned to its previous low level during the relapse of one patient following withholding of PGA, and again rose after a second period of therapy with PGA.

Following therapy with PGA an improved ability to absorb vitamin A is indicated by a rise in serum vitamin A following the administration of an oral test dose. The fact that this curve reverted to a typical flat curve during a relapse after the withdrawal of treatment is further evidence of the influence of this factor on the absorption of fat-soluble substances.

It is suggested that the M group of vitamins plays an important role in the normal physiology of the gastrointestinal tract.

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